JOURNAL OF DAIRY SCIENCE

Contents

Comparison of Limestone and Sodium Bicarbonate as Neutralizers for Phosphoric Acid Oat Silage. W. A. KING 975

The Relation of Longevity to Fertility of Bull Semen. S. MARGOLIN, J. W. BARTLETT and O. L. LEPARD 983

A Study of the Blood Carotene in Relation to Lipolytic Activity of Milk. N. P. TARASSUK and W. M. REGAN 987

Homogenizing Efficiency Test Studies. H. F. JUDKINS 997

A Method of Equalized Feeding for Studies with Dairy Cows. H. L. LUCAS 1011

Certain Dietary Factors Essential for the Growing Calf. NORMAN S. LUNDQUIST and PAUL H. PHILLIPS 1023

Observations on the Ascorbic Acid Content of Evaporated Milk F. J. DOAN and D. V. JOSEPHSON 1031

Rennin Action in Relation to the Water Binding and Electrokinetic Properties of Calcium and Sodium Caseinate Sols. C. L. HANKINSON and L. S. PALMER 1043

The Effect of Dilution Rate on the Livability and the Fertility of Bull Spermatozoa Used for Artificial Insemination. G. W. SALISBURY, G. H. BECK, P. T. CUPPS and IRVINE ELLIOTT 1057

Announcement 1070

Abstracts of Literature A213

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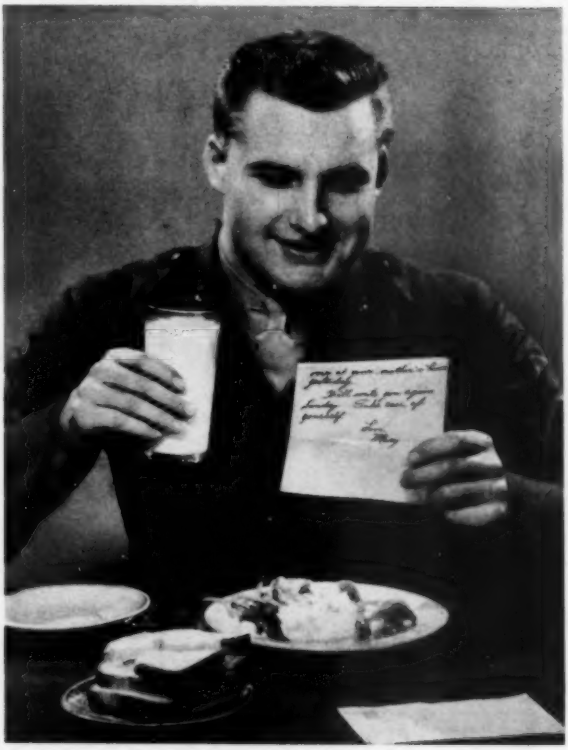

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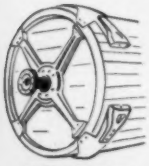

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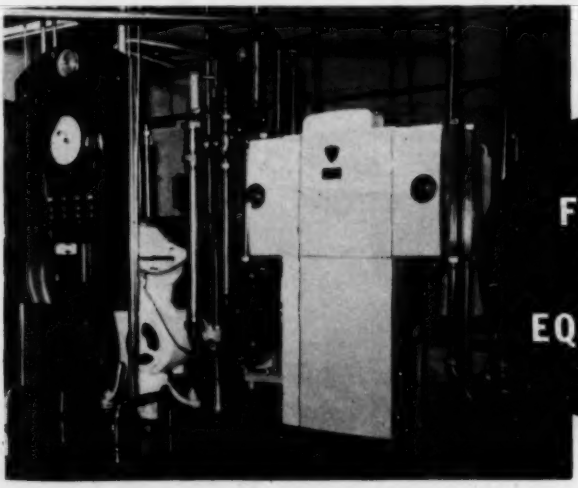

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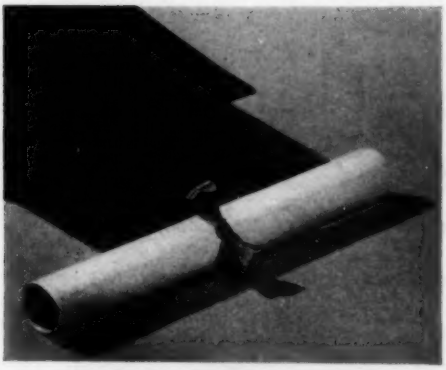

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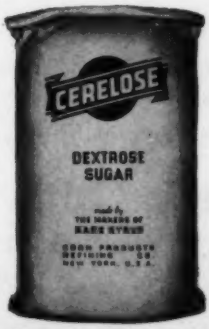

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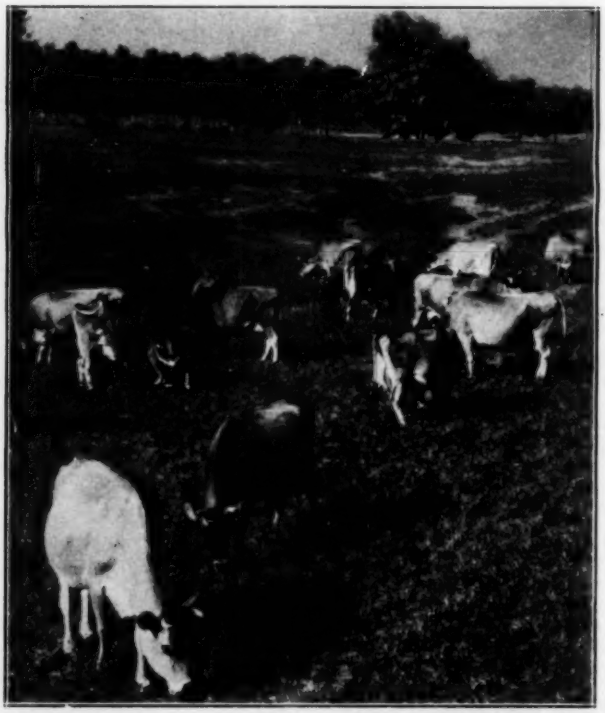

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JOURNAL OF DAIRY SCIENCE

VOLUME XXVI NOVEMBER, 1943 NUMBER 11

COMPARISON OF LIMESTONE AND SODIUM BICARBONATE AS NEUTRALIZERS FOR PHOSPHORIC ACID OAT SILAGE*

W. A. KING

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Ever since mineral acids became popular as preservatives for silage it has been customary to feed neutralizers such as limestone and sodium bicarbonate with the mineral acid silages. Limestone has been the most commonly used, mainly because of its low cost and its calcium content. Both limestone and sodium bicarbonate have been used in many experiments (1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 13) to neutralize the mineral acids without too much distinction between their effectiveness as neutralizing agents. There is some evidence in the literature (3, 4, 7, 11), however, that limestone is not as efficient a neutralizer as sodium bicarbonate. Brouwer (3) found sodium bicarbonate to be twice as efficient as limestone for the neutralization of A.I.V. acid silage fed to milking cows, and Rottensten (11) observed that limestone only partly counteracted the acid action of the A.I.V. silage fed to steers, whereas sodium bicarbonate was much more effective. Mollgaard and Thorbek (7) fed A.I.V. acid silage to cattle with and without neutralizers and observed that sodium bicarbonate counteracted the acid of the silage completely, whereas limestone did not in the amounts which they fed. However, a serious objection to the use of limestone or sodium bicarbonate for neutralizing the acid, was that a progressive fall in the digestibility of the silage nutrients, particularly the crude protein, occurred upon partial neutralization by limestone and upon complete neutralization by sodium bicarbonate. The metabolizable energy likewise decreased. The feeding of beet roots was recommended, since the acid was thus neutralized satisfac-

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* Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 221. This project was under the direction of a committee of the Departments of Agricultural Biochemistry and Dairy Husbandry consisting of W. C. Russell (Agricultural Biochemistry), Chairman, J. W. Bartlett (Dairy), C. B. Bender (Dairy), O. F. Garrett (Dairy), O. L. Lepard (Dairy) and H. J. Metzger (Agricultural Biochemistry).

Journal Series paper of the New Jersey Agricultural Experiment Station, Rutgers University, Departments of Dairy Husbandry and Agricultural Biochemistry.

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torily and no depression of digestibility took place. Eaton (4) found that limestone counteracted the acid of phosphoric acid oat and pea silage satisfactorily but not that of phosphoric acid oat silage. Limestone and sodium bicarbonate fed together in equivalent amounts to neutralize two hydrogens of the acid in the phosphoric acid oat silage completely maintained normal acid-base equilibrium of heifers, and mixed hay, also, showed some value in this respect. Lepard *et al.* (6) reported that either a legume hay or limestone could be used to maintain a normal condition when phosphoric acid grass silages were fed. Perkins and Monroe (9) observed that alfalfa hay was much more effective than ground limestone in restoring the urinary reactions to normal when A.I.V. acid silage was fed. Furthermore, work at this Station (5) indicated that additions of limestone counteracted the acid in phosphoric acid alfalfa silage fed to milking cows. The above investigations indicate that legumes, whether in hay or in silage, have a definite neutralizing effect when acid silage is fed, and that sodium bicarbonate is more effective than limestone in neutralizing the acid of the mineral acid silages.

EXPERIMENTAL

The experiments here reported ran concurrent with a series of experiments in which the nutritive value of the oat plant, as a silage in a dairy cow ration, was being investigated. The animals were fed only phosphoric acid oat silage and salt, and a neutralizer as indicated, or, as in the case of three cows, the silage was supplemented with mixed alfalfa-timothy hay and a grain mixture. Because the cows that were fed the silage alone could not consume sufficient nutrients for both maintenance and good milk production, the average production was only about 15 pounds but the animals remained in fair condition. The first part of the experiment was conducted during the winter of 1941–42 and the remainder during the following summer on the same silage. Both the limestone and the sodium bicarbonate were commercial products.

The oat silage was preserved with 20 pounds of 68 per cent phosphoric acid per ton of green material, the acid being diluted with an equal weight of water to assure more uniform distribution. The oats were in the milk stage at the time of ensiling and the silage was of good quality.

The CO_2-combining capacity of the blood plasma and the fixed CO_2 of the urine were determined with a Van Slyke manometric gas apparatus (10). The pH of the urine was determined with a Beckman glass electrode pH meter and the urinary ammonia by Folin's aeration method as modified by Steele (12). In every case the urine samples were a portion of a 24-hour collection. The blood and urine examinations were made at various intervals throughout the experiment as indicated.

RESULTS

A preliminary period of two weeks was used in which the ration was changed from that of the milking herd to the experimental ration. The values (table 1) expressed at zero days of experiment are actually averages

TABLE 1

Physiological reactions of cows fed phosphoric acid oat silage with and without neutralizers

Days of exp.	403				435				437			
	Plasma CO_2 – comb. cap.	Urine (24-hr. sample)			Plasma CO_2 – comb. cap.	Urine (24-hr. sample)			Plasma CO_2 – comb. cap.	Urine (24-hr. sample)		
		pH	NH_3	Fixed CO_2		pH	NH_3	Fixed CO_2		pH	NH_3	Fixed CO_2
	vol. %		*mg.*	*mM*	*vol. %*		*mg.*	*mM*	*vol. %*		*mg.*	*mM*
	No neutralizer				No neutralizer				No neutralizer			
0	51.7	7.96	138	811	50.1	8.05	60	377	55.3	7.97	135	259
5	.....	5.50	524	36	.....	5.96	164	22	.....	5.66	267	12
11	42.1	5.82	1885	15	32.1	5.63	1347	20	42.3	5.69	2805	13
20	40.6	5.92	2240	27	28.2	5.95	1872	24	36.8	5.84	2001	20
	$CaCO_3$ – 2H				$CaCO_3$ – 2H				$CaCO_3$ – 2H			
41	48.4	6.48	784	66	46.5	5.90	894	12	51.2	5.84	882	13
56	51.9	5.36	862	22	48.0	5.75	2078	20	57.3	5.62	1266	7
	$CaCO_3$ – 3H								$CaCO_3$ – 1H; $NaHCO_3$ – 1H			
65	56.7	7.94	83	840	49.8	5.74	489	17	65.9	7.82	32	793
					$CaCO_3$ – 3H				$CaCO_3$ – 3H			
75	.....	7.89	11	459	.....	7.82	16	741	.....	8.08	24	512
86	56.4	7.03	285	147	44.7	7.42	69	384	59.6	8.01	93	351
	$CaCO_3$ – 2H				$CaCO_3$ – 2H				$CaCO_3$ – 2H			
97	.....	7.68	359	392	.....	7.35	32	279	.....	7.55	83	167
107	44.1	5.08	1332	25	36.1	5.55	2742	43	38.2	5.25	1210	19
114	.....	5.24	1404	20	.....	5.65	2507	21	.....	5.25	855	17
121	53.3	5.10	230	11	41.9	5.38	551	11	53.0	5.20	1174	12
					$CaCO_3$ – 1H; $NaHCO_3$ – 1H				$NaHCO_3$ – 1H			
128	48.0	5.12	1306	22	53.5	6.62	49	83	55.0	5.51	339	13
142	.....	5.22	1807	25	.....	7.48	155	531	.....	6.11	160	18
149	46.8	6.19	228	27	61.8	7.50	99	509	50.5	6.58	198	31

of two different examinations of the cows' blood and urine while the herd ration was being fed. The different neutralizers with the amounts fed are shown in tables 1 and 2. For example, $CaCO_3$ – 2H means that ground limestone was fed in sufficient amounts to neutralize two hydrogens of the phosphoric acid that was present in the silage consumed.

For the first 28 days, including the preliminary period, the phosphoric acid oat silage was fed alone. The progressive effect of the acid silage on all three animals, 403, 435, and 437, was very evident in both urine and blood

(table 1). The pH of the urine dropped from an average of 8 to 5.9, the ammonia excretion increased from an average of 111 mg. per 24 hours to over 2000 mg. and the fixed CO_2 decreased from 482 mM to about 20 mM per 24 hours. The drop in the CO_2-combining capacity of the blood plasma averaged 17.3 volumes per cent. These reactions of the cows to the acid silage were large and a decided change in appearance of the animals was soon evident. The cows consumed less than 50 pounds of silage per day (one as low as 30 pounds) and there was an average loss of 138 pounds of live weight, along with an 18-pound drop in milk production. Some of this response, but not all, can be explained by the insufficient consumption of nutrients.

TABLE 2

Physiological reactions of cows fed phosphoric acid oat silage with and without sodium bicarbonate

Days of exp.	416				427				468			
	Plasma CO_2 – comb. cap.	Urine (24-hr. sample)			Plasma CO_2 – comb. cap.	Urine (24-hr. sample)			Plasma CO_2 – comb. cap.	Urine (24-hr. sample)		
		pH	NH_3	Fixed CO_2		pH	NH_3	Fixed CO_2		pH	NH_3	Fixed CO_2
	vol. %		*mg.*	*mM*	*vol. %*		*mg.*	*mM*	*vol. %*		*mg.*	*mM*
	No neutralizer				No neutralizer				No neutralizer			
0	52.5	8.15	58	1644	51.3	8.17	194	1902	53.0	7.92	50	1862
7	46.0	5.15	4210	9	45.0	5.80	5916	44	42.0	5.45	5774	15
14	41.6	4.92	2438	4	38.6	5.18	3416	3	45.3	5.25	5354	6
	$NaHCO_3$ – 1H				$NaHCO_3$ – 1H							
21	51.0	5.65	734	14	46.8	5.83	415	20	39.4	5.29	6119	5
28	54.5	5.63	381	7	50.8	5.95	364	11	32.4	5.30	4904	2
	$NaHCO_3$ – 2H				$NaHCO_3$ – 2H				$NaHCO_3$ – 2H			
35	54.4	7.73	83	580	57.4	7.80	73	574	57.6	8.20	42	1227

At the end of the 28-day period it was decided to supplement the ration with limestone sufficient to neutralize two hydrogens of the phosphoric acid in the silage. The most obvious result was the improvement in general appearance. The silage consumption increased and the cows gained some weight. The CO_2-combining capacity of the blood plasma returned essentially to the previous normal level and some temporary improvement in the urine was evident. After 28 days (56th day of experiment) of this regime, a quantity of limestone sufficient to neutralize all three hydrogens of the phosphoric acid was fed to cow 403, cow 435 was continued as before as a control animal, and cow 437 was fed limestone and sodium bicarbonate in chemically equivalent amounts to neutralize two hydrogens. Cows 403 and 437 showed an immediate response. The urine of both showed normal acid-base equilibrium nine days later (65th day of experiment) and the CO_2-combining capacity of the plasma had increased, especially in 437.

Since the response of cow 403 indicated that limestone sufficient to neutralize all three hydrogens of the phosphoric acid in the oat silage was necessary to counteract the acid, it was decided that all three cows should be fed this amount for the next 21 days during which time collections were to be made for digestion and balance studies. These additional studies were concluded on the 86th day of experiment. During this latter period the blood and urine of cows 403 and 437 remained normal, the urine of cow 435 returned to normal but her blood plasma showed a decrease in the CO_2-combining capacity. In view of the urine being normal this plasma value seems to be an error. Amounts as high as 12 ounces of limestone per day were fed in this period to cow 435.

Previous trials had shown that limestone fed in sufficient quantity to neutralize two hydrogens of the phosphoric acid in the oat silage was not very efficient in correcting the acid condition of the urine in a curative role, but it was thought that limestone in similar amounts might prove more beneficial if it were fed as a preventive. This measure was tried with all three cows for 35 days from the 86th to 121st day of experiment. Here again limestone failed to prevent the acid condition that soon became apparent in the urine. There was a lag period, however, of at least 21 days before this condition appeared, possibly due to a residual effect of the feeding of a larger quantity of limestone in the preceding period.

Inasmuch as good results had been obtained with supplements of sodium bicarbonate in a previous period it was decided to study further the effectiveness of this compound. With cow 403 left as a control animal on the limestone sufficient to neutralize two hydrogens, cow 435 was fed limestone and sodium bicarbonate in equivalent amounts to neutralize two hydrogens of the phosphoric acid in the oat silage and cow 437 was fed sodium bicarbonate to neutralize one hydrogen. Again, the combination of limestone and sodium bicarbonate fed to cow 435 proved more efficacious than limestone sufficient to neutralize three hydrogens. Sodium bicarbonate improved the condition of the urine of cow 437 somewhat over the "2 hydrogen-limestone" basis.

To supplement further the study of the value of sodium bicarbonate, three more cows were fed phosphoric acid oat silage alone for 14 days and at the end of this period all three showed a definite acid condition of the blood and urine (table 2). At this time the rations of two cows, 416 and 427, were supplemented with sufficient sodium bicarbonate to neutralize one hydrogen of the acid, while cow 468 was continued as a control animal. There was an immediate return toward normal of the ammonia and pH of the urine of cows 416 and 427, but the change in fixed CO_2 was not marked. The CO_2-combining capacity of the blood plasma returned to normal. Even so, this showed that the results were as good as, or better than, those obtained when sufficient limestone was fed to neutralize two hydrogens of the

phosphoric acid. After being fed sodium bicarbonate on this basis for 14 days, each of the three cows was given a quantity of sodium bicarbonate sufficient to neutralize two hydrogens of the acid. The response was immediate and complete. The blood and urine examinations seven days later showed completely normal acid-base equilibrium.

Attention should be directed to the high acid reaction, pH 4.92, of the urine of cow 416 at 14 days of experiment. Also the ammonia excretion values of 5916 and 6119 mg. per 24 hours of cows 427 and 468 respectively are high.

Another interesting observation was made on an additional group of three cows (table 3) which received phosphoric acid oat silage supplemented

TABLE 3

Physiological reactions of cows fed phosphoric acid oat silage supplemented with mixed hay and 2 per cent limestone in the grain mixture

Days of exp.	392				423				426			
	Plasma CO_2–comb. cap.	Urine (24-hr. sample)			Plasma CO_2–comb. cap.	Urine (24-hr. sample)			Plasma CO_2–comb. cap.	Urine (24-hr. sample)		
		pH	NH_3	Fixed CO_2		pH	NH_3	Fixed CO_2		pH	NH_3	Fixed CO_2
	vol. %		*mg.*	*mM*	*vol. %*		*mg.*	*mM*	*vol. %*		*mg.*	*mM*
0	56.1	7.62	120	334	53.2	7.32	167	216	54.5	7.20	135	106
9	57.6	7.29	169	146	55.8	7.27	117	126	61.1	6.08	267	15
23	61.9	7.24	87	108	51.1	6.41	139	25	59.6	6.98	97	73
44	56.8	6.76	234	83	51.2	6.02	338	21	63.4	7.37	456	75
65	.....	7.39	89	335	.....	7.79	73	361	.....	7.85	88	356
86	53.5	6.10	869	30	52.1	5.70	850	17	60.0	5.97	266	28
107	49.2	5.80	457	23	46.8	5.66	524	16	51.3	5.78	496	15
128	49.9	5.97	1376	17	42.0	5.79	1293	18	47.6	5.88	1356	22

with hay and a grain mixture. The grain mixture contained two per cent limestone and the hay was good quality alfalfa-timothy mixed hay. The limestone consumed was sufficient to neutralize 1.6 hydrogens of the phosphoric acid in the silage. After 86 days of experiment, a definite increase in urinary ammonia and a decrease in fixed CO_2 and in the pH of the urine were evident. By the end of the experiment, at 128 days, urine examinations showed that the cows were in a condition almost as bad as any of the previously described animals before the addition of a neutralizer was made. The CO_2-combining capacity of the plasma also showed a definite acid condition but not to as great an extent as when no neutralizer was fed.

SUMMARY

1. Limestone and sodium bicarbonate were compared as neutralizers when fed with oat silage preserved with 20 pounds of 68 per cent phosphoric acid per ton of green material. Sodium bicarbonate proved to be considerably more efficacious than limestone.

2. Sodium bicarbonate was about twice as efficient as limestone in counteracting the acid in phosphoric acid oat silage. On the two-hydrogens-neutralized basis, limestone was not completely effective in either curative or preventive trials, whereas the quantity of sodium bicarbonate sufficient to neutralize one hydrogen of the acid in the phosphoric acid oat silage proved to be slightly superior. Sodium bicarbonate and limestone when fed in equivalent amounts to neutralize two hydrogens of the phosphoric acid were adequate, and this combination is probably the most satisfactory for practical purposes. Sodium bicarbonate when fed on the two-hydrogens-neutralized basis proved to be efficient.

3. There is apparently a relation between the acid condition of the animals and the amount of silage consumed, because after adequate neutralization of the acid the silage consumption increased rapidly.

4. A grain mixture containing two per cent limestone along with a mixed alfalfa-timothy hay of good quality proved to be ineffective in preventing acidosis in cows fed phosphoric acid oat silage in a long-time feeding experiment.

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THE RELATION OF LONGEVITY TO FERTILITY OF BULL SEMEN*

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Several criteria have been used by investigators in determining the fertility of semen. These criteria include initial motility, concentration, morphology, percentage of abnormal sperm, pH, volume, longevity (1–10), respiration rate (9), and glycolytic activity (1). Such characteristics have been used individually and in combination. Only the respiration rate has been shown to have a highly significant relation to fertility. All others with the exception of longevity and glycolytic activity have been conclusively found by various workers (2, 3, 7, 8, 9) to be inadequate measurements of fertility. Herman and Swanson (3) and Comstock (1) have mentioned a possible significant relationship between longevity (duration of motility) and fertility.

Records available through the cooperation of the New Jersey Artificial Breeding Unit No. 2 with the New Jersey Agricultural Experiment Station provided data suitable for a study of the relationship of breeding efficiency and semen longevity. Data were kept of the longevity of 207 "normal" semen samples collected from ten sires over a period of one year. Semen samples were diluted with egg-yolk phosphate buffer (5), and a portion was placed in a refrigerator at 45° F. to determine its longevity. Motility estimations were made at 24-hour intervals until motility ceased.

There were 483 services with fresh and one-day semen[1] to 306 cows before all conceived. Only services with fresh and one-day semen were used because they had a very similar conception rate, 63.7 per cent and 61.9 per cent, respectively. Cows requiring more than three services of fresh or one-day semen per conception were omitted in the tabulation of the data in order to minimize the influence of "non-breeders" upon the estimation of the fertility of any given semen sample. Inseminations were made by the veterinarians of the artificial breeding association.[2] Approximately 0.5 cc. of diluted semen was used for each insemination. Accurate records were kept of the sire used, date of service, age of semen, and the number of services required per conception for each cow. Conception was ascertained by palpation or by the calving of the cows served.

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[1] Fresh semen refers to semen less than 24 hours old; one-day semen is more than 24 hours, and less than 48 hours old.

[2] The authors wish to acknowledge the assistance of Dr. D. Master, Dr. E. J. Weatherby, and Mr. C. E. Shuart, whose cooperation made this study possible.

The breeding record for each semen sample was established, and the mean conception rate for each sample was determined. These individual mean conception rates were tabulated according to the longevity of the sample. The mean conception rate for the respective longevity classes are indicated in table 1.

TABLE 1

The relation of longevity to percentage of conception

Longevity (days)	Number of samples	Number of services	Percentage of conception
14 or more	3	11	88.9
13	12	34	65.0
12	25	56	62.3
11	34	81	56.3
10	52	127	68.6
9	36	74	63.6
8	21	53	58.6
7	14	27	40.4
6 or less	10	20	35.0
Total or mean	207	483	62.3

The correlation coefficient between longevity in days and the mean conception rate (table 1) was significant, $+0.6964 \pm 0.0212$.

The correlation of longevity with percentage conception for each sire is shown in table 2. Six of the sires show a significant positive correlation, and two sires have negative correlations of low significance. Two sires, 100B and 100E, had only two and five samples in the data, and as a result are not included in this table. It will also be noted that sires with the highest mean conception rates had the most significant correlation coefficients.

TABLE 2

Correlation coefficients of longevity vs. percentage of conception by sires

Sire	Mean percentage of conception	Number of samples	Coefficient
E	60.7	39	-0.1600 ± 0.1346
F	65.4	45	$+0.4134 \pm 0.0874$
T	67.8	29	$+0.7287 \pm 0.0513$
X	75.0	13	$+0.9455 \pm 0.0157$
100A	47.9	33	-0.4049 ± 0.1051
100G	55.6	9	$+0.5623 \pm 0.1548$
100X	65.8	12	$+0.8930 \pm 0.0323$
100Z	71.7	20	$+0.6224 \pm 0.0866$

The dislocation from the statistical regression line by the mean conception rates for semen samples of some longevities (table 1) is attributed to inadequate sampling. The total sample was too small, and furthermore, the number of cows served per sample varied considerably (one to seven services per sample). This latter factor was particularly true of the sires

with the insignificant negative correlation coefficients. The mean deviation of the number of services per cow for these two sires was high compared to that of the remaining sires.

SUMMARY

1. The analysis of the breeding records kept on ten sires and 306 cows show a highly significant correlation between the longevity and the conception rate of bull semen ($+0.6964 \pm 0.0212$).

2. Six of eight sires studied showed significant positive correlations between longevity and conception rate; two showed insignificant negative correlations.

3. There was only a slight difference in percentage of conception for fresh semen and one-day semen (63.7 per cent and 61.9 per cent, respectively).

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A STUDY OF THE BLOOD CAROTENE IN RELATION TO LIPOLYTIC ACTIVITY OF MILK

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Hydrolytic rancidity in milk results from hydrolysis of the milk fat, a reaction activated by the enzyme lipase. According to the present view there are two kinds of lipolytic action: the induced form initiated by modification of the surface of the fat globules, and the spontaneous type that proceeds naturally with the aging of the cooled milk (16). It is with the latter type that this paper is concerned.

As early as 1909 Paraschtschuck (14), who first detected a naturally-active lipase in cow's milk, reported that the amount present increases at the end of the lactation period. Other workers have amply confirmed (1, 5, 8, 9, 10, 12) the observation that the development of rancid flavor is most common in late lactation. Apparently another cause for increased concentration of naturally active lipase is the absence of green feed. According to Csiszar (4), Hileman and Courtney (8), Fouts and Weaver (6), and others, rancid flavor is at a minimum during spring and summer, when the cows are on pasture. In 1936 Anderson (2) concluded, after observing two herds and collecting data on one cow, that a deficiency of certain dietary factors causes the secretion of milk that becomes rancid. When he substituted 10 pounds of artificially dried hay for an equal amount of field-cured hay in a ration of 25 pounds of corn silage, 10 pounds of hay, and 9 pounds of grain, the production of rancid milk ceased in 3 or 4 days. Results were even more striking when 5 pounds of carrots was used to replace the hay. The feeding of vitamin A, though somewhat less effective, also lessened the degree of rancidity. Though Anderson did not study the carotene content of the milk, he designates a deficiency of carotene in the diet as the probable causative factor.

Tarassuk, in 1939, (15) reported a case history strongly indicating the presence of an anti-lipolytic principle in green feed. A cow late in lactation, on a ration of hay and concentrates, was producing rancid milk. When green feed was substituted for hay, the rancidity no longer developed. Upon a return to the hay and grain ration, however, lipolytic activity was restored to the previous level.

To shed more light on the principle responsible for the beneficial effect of "green feed" in preventing lipolysis, and to establish whether carotene

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is the specific factor involved—as Anderson (2), Anderson, Hardenbergh, and Wilson (3) have suggested—the authors have studied the relation between the carotene content of the ration and the development of spontaneous rancidity.

EXPERIMENTAL

In May, 1940, three Jersey (588, 569, and 707) and one Guernsey (620) cows were taken from alfalfa pasture and fed bleached alfalfa hay and concentrates. The carotene content of this basal ration was very low—only 0.05 mg. per 100 grams of feed.

Before the cows were put on experimental regime, their milk was tested for gross composition, pH, stability to heat, rennet coagulation, and flavor; it proved normal in all respects. For a while during the previous winter, cow No. 588 was known to have secreted milk that would go rancid spontaneously upon aging.

Throughout the 190 days of the main experiment, the milk was sampled daily. The samples were cooled in ice-cold water within one hour after milking and were aged at 5 to 7° C. The extent of lipolysis was ascertained 1) by measuring the surface tension of milk at 1, 2, and 7 days of aging and 2) by determining weekly the free fatty acids of milk fat in samples aged for 7 days. Periodically the milk samples were also scored for flavor and odor. At frequent intervals the blood-carotene level and the carotene content of milk fat were determined for each cow. Carotene in the blood[1] was determined with a photoelectric colorimeter, according to a method described by Moore (11).

The carotene in milk fat was determined with a Coleman Universal Spectrophotometer (Model 11), petroleum ether being used as the solvent. Crystalline β-carotene was employed for standardizing the instrument and for plotting the standard curve. These determinations were made on aliquots of the same samples of pure, dry fat used for determining free fatty acids. Figures 1, 2, 3, and 4 show the feeding regimes imposed and the results obtained.

As mentioned before, the basal ration (bleached alfalfa hay and concentrates), contained but 0.05 mg. of carotene per 100 grams of feed. Whenever supplementary carotene was fed in the form of crystalline β-carotene dissolved in cottonseed oil, or in green alfalfa, it is indicated on the curves.

Figure 1 shows the blood-carotene content during the first 190 days of experiment. On the basal ration of bleached hay and concentrates, the blood-carotene level for all cows declined rapidly from the starting value of 1.42 to 2.31 mg. per 100 cc. plasma to less than 0.25 mg. per 100 cc. plasma at the end of 30 to 40 days. Thereafter it became stabilized, leveling off near 0.1 mg. per 100 cc. plasma, until the diet was supplemented with addi-

[1] Carotene in the blood was determined by H. Goss and S. W. Mead of the Animal Husbandry Division.

tional carotene. A more detailed picture of blood-carotene study in this experiment has been presented by Goss and Mead (7). Oral administration of 250 mg. of crystalline carotene in cottonseed oil only slightly increased the blood carotene. An addition to the basal ration of 500 mg. of crystalline carotene in cottonseed oil markedly increased the blood-carotene level. When 1000 mg. of carotene was fed, the repletion of blood carotene proceeded rapidly, at a rate similar to that of the depletion at the beginning of the experiment.

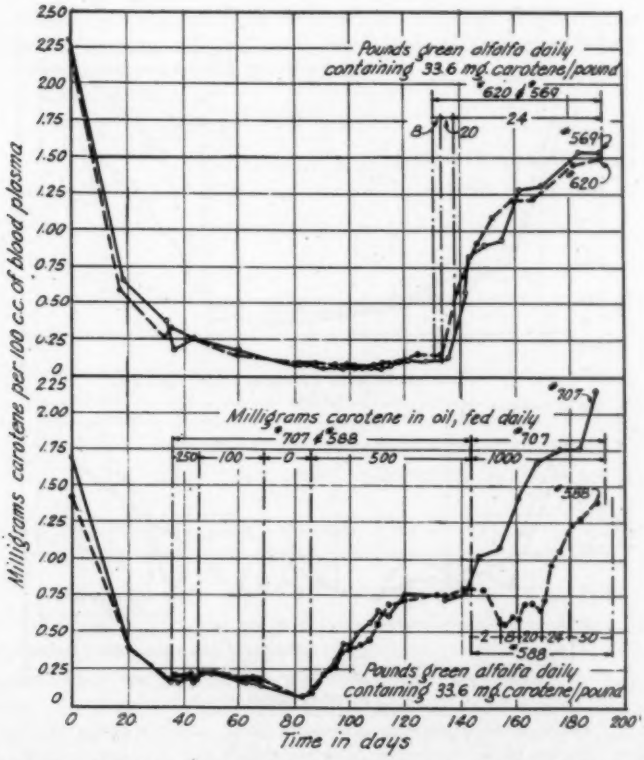

FIG. 1. The depletion of carotene in the blood of cows on a diet of bleached alfalfa hay, and its repletion by feeding of (a) green alfalfa and (b) carotene in oil (courtesy of Mead and Goss).

Figure 2 presents the carotene content of milk fat. On comparing figures 1 and 2 one sees, as would be expected (13), a direct relation between the concentration of carotene in the blood and in milk fat. Furthermore, not only does the carotene content of each cow's milk fat show the same

trends as her blood carotene, but the numerical values expressed as mg. of carotene per 100 cc. of blood plasma and mg. of carotene per 100 cc. of milk fat are nearly the same for any particular time.

Figures 3 and 4 show the extent of spontaneous lipolysis of milk from these 4 cows on a low- and high-carotene feeding regime. These figures give the surface tension of the milk of each cow from the beginning of the experiment until the cow was dried. The surface-tension data are the values obtained on the samples of milk cooled and aged for 48 hours. Surface-

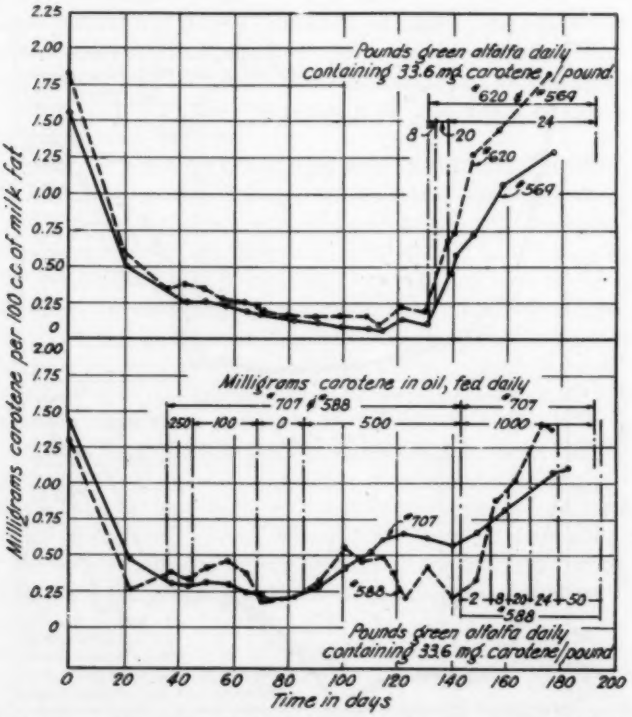
Milligrams carotene per 100 c.c. of milk fat
Pounds green alfalfa daily containing 33.6 mg. carotene / pound
Milligrams carotene in oil, fed daily
Pounds green alfalfa daily containing 33.6 mg. carotene/pound
Time in days

FIG. 2. The depletion of carotene in the milk fat of cows on a diet of bleached alfalfa hay, and its repletion by the feeding of (a) green alfalfa and (b) carotene in oil.

tension values below 46 dynes per cm. are characteristic of rancid milk. The lower the surface tension, the greater the extent of hydrolytic rancidity, as Tarassuk (15) has pointed out.

The data on cow 588 show strikingly the beneficial effect of green feed in preventing lipolysis of fat, yet the independency of lipolysis from the amount of carotene in the cow's blood and milk fat. At about the time when her blood carotene reached the minimum level, her milk upon aging 48 hours

showed slight rancidity. It became progressively more rancid despite the increase in blood carotene that was caused by the feeding first of crystalline β-carotene and later of green feed up to 24 pounds daily. Between the 90th and the 176th day of the experiment, the surface tension of No. 588's milk dropped from 45 dynes per cm. to 33.9 dynes per cm. In the same period the level of her blood carotene increased steadily from 0.20 mg. per 100 cc. of plasma to 1.04 mg. per 100 cc. The rapid drop of surface-tension values occurs during the advanced stage of lactation. On the 160th day of the experiment, this cow was due to calve in about 9 weeks. At this advanced stage of lactation the lipolysis of milk fat increased rapidly despite the ingesting of some green feed. On the 179th day of the experiment the

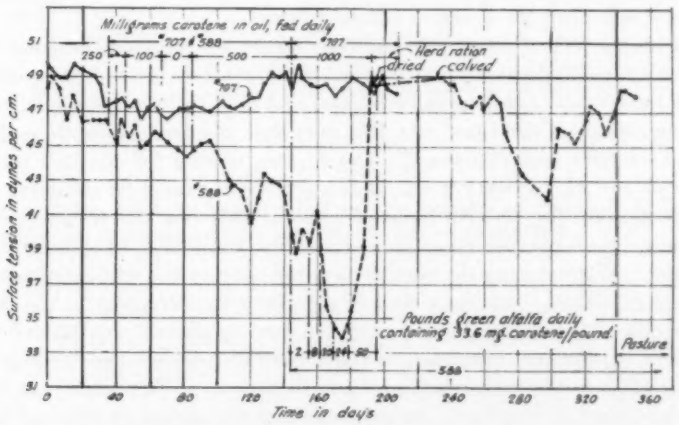

FIG. 3. The extent of hydrolytic rancidity in the milk of cows on a diet of bleached alfalfa hay and on bleached alfalfa hay supplemented by (a) carotene in oil and (b) green alfalfa.

amount of green alfalfa fed daily was increased to 50 pounds; and in about 10 days her milk became normal in lipolytic activity, as was evidenced by a sharp rise of its surface-tension values. The recovery is remarkable, occurring when the cow was very late in lactation and was producing milk extremely high in lipolytic activity. She was due to calve in about 40 days. On the 196th day of the experiment she was dried and put back on the basal diet to show whether, under this condition, she would produce a rancid milk after parturition. When she freshened, 4½ weeks later, the surface tension of her milk was between 49 and 47 dynes per cm. for the first month of lactation. Later it fell below 46 dynes per cm. Her milk never again became so rancid as when she was late in lactation. During this period the lowest surface-tension value was 42 dynes per cm. On the 109th day after freshening she was put on pasture, and her milk became normal.

Cow 707 was on exactly the same feeding regime as No. 588 for the first 146 days of the experiment. From the 146th to the 195th day her basal ration was supplemented with 1000 mg. of crystalline β-carotene daily; and later, until she was dried, she was on the herd's winter ration. Her blood level of carotene was almost identical with that of cow 588, except for a more rapid increase of blood carotene toward the end of the experiment. Yet her milk never became rancid. Instead, after she had been on the basal diet about 20 days, her milk upon aging 48 hours became spontaneously oxidized. The daily feeding of 1000 mg. of crystalline β-carotene reduced the tendency to oxidize spontaneously, but did not eliminate this defect entirely when milk was aged longer than 48 hours. Nor did the herd's ration, consisting of good-quality dry hay and concentrates, entirely eliminate oxidized flavor. The cow was dried but a month before calving.

The other pair of cows—620 and 569 (figure 4)—were on the same feeding regime throughout. They received the basal diet for 132 days, during which the levels of carotene in both blood and milk fat were very low, under 0.20 mg. most of the time. At this very low carotene level, the surface tension of their milk decreased. The surface tension for No. 620 was between 47 and 44.5 dynes per cm., indicating slight rancidity at times. The surface tension for No. 569 hovered between 46.0 and 48.0 dynes per cm. Her milk, besides bordering often on rancidity, would also be spontaneously oxidized. When during the next 56 days the basal ration was supplemented with green alfalfa, both cows showed a marked improvement in the flavor of the milk. At this time both were due to calve in about 2 months, and they were put back on the basal diet to show whether bleached-hay feeding and late lactation would cause the high lipolytic activity. This effect was strikingly produced in cow 620. As she came into the advanced stage of lactation, her milk production dropped. The surface tension began to decline even while she was fed 24 pounds of green alfalfa daily, and fell abruptly after the complete change to a basal diet. The same feeding regime and similar stages of lactation produced no changes in the milk of No. 569. Later, however, this animal proved not to be pregnant; she was a nonbreeder.

Figure 5 presents further evidence that carotene deficiency in the diet will not, *per se*, cause a cow to secrete milk that will spontaneously go rancid.

Cows 730 and 712 were put on bleached alfalfa and concentrates in their second month of lactation. They remained on this low carotene diet throughout the lactation period. In 40 days the carotene in their blood and milk fat dropped from the values above 1 mg. to about 0.20 mg. per 100 cc. of blood plasma or milk fat. After 3 months on bleached hay the level of carotene for No. 730 leveled off between 0.04–0.05 mg., that for No. 712 between 0.07 and 0.10 mg. of carotene per 100 cc. of blood plasma.

The milk of No. 712 became slightly rancid for about 20 days between the 15th and 40th day of experiment, as was evidenced by a decrease in its

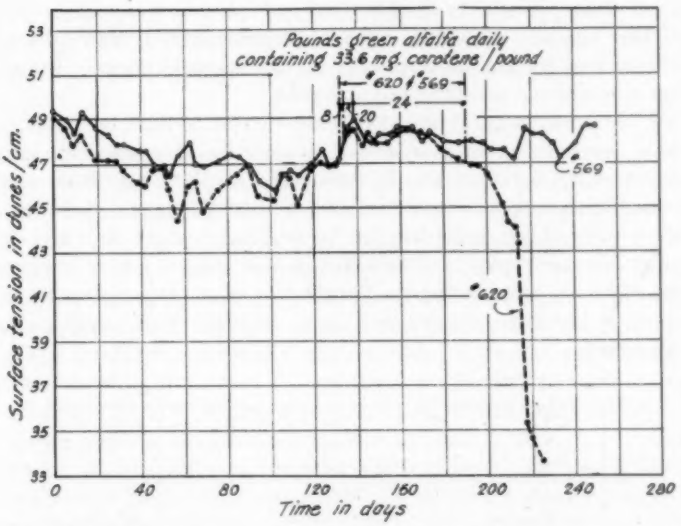

FIG. 4. The extent of hydrolytic rancidity in the milk of cows kept on bleached alfalfa hay and on bleached alfalfa hay supplemented by green alfalfa.

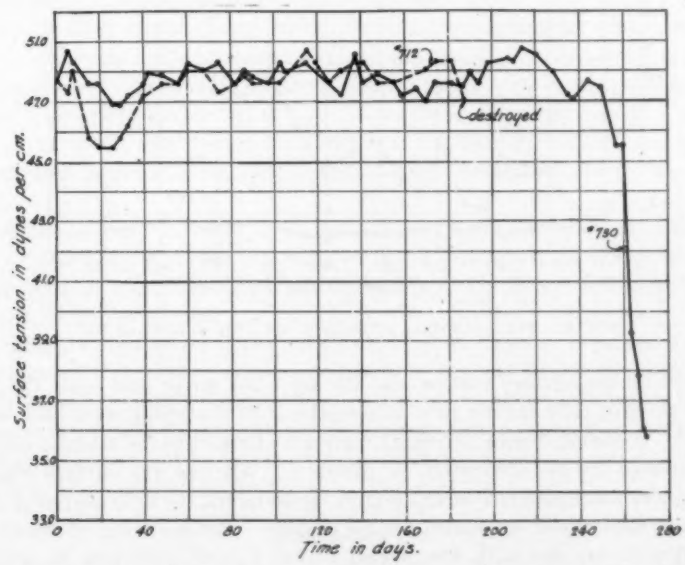

FIG. 5. The extent of hydrolytic rancidity in the milk of cows on a diet of bleached alfalfa hay.

surface tension. While the level of her blood carotene was still going down, the surface tension of her milk went up, to remain well above the border line of rancidity for the rest of the 149 days of the experiment. Toward the end her milk became spontaneously oxidized.

Cow 730 presents an illuminating case. After 34 days on the low-carotene diet, her milk upon aging 48 hours would be spontaneously oxidized. She continued to secrete such milk, free from hydrolytic rancidity, until she had been 250 days on the basal diet and in milk 317 days. In 10 days the surface tension of her milk dropped below 40 dynes per cm.; and it continued to decrease rapidly. Although this cow was on a very low-carotene diet for 270 days, only during the last 20 days of the experiment, in a very late stage of lactation, did she start to secrete milk that would go rancid spontaneously.

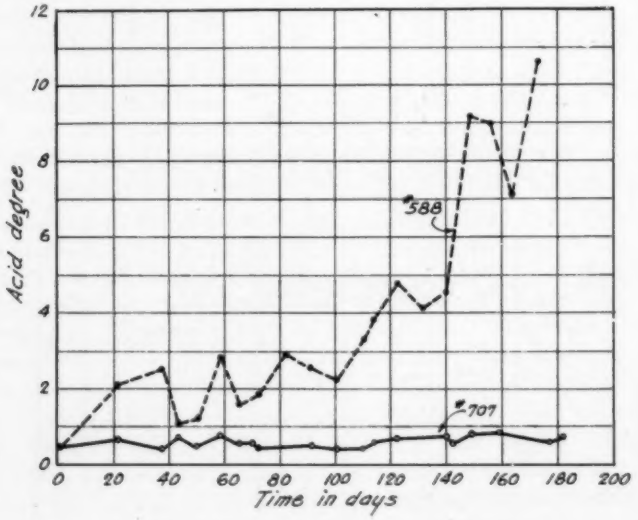

FIG. 6. The acid degree of the milk fat of cows 588 and 707.

Besides the surface tension and the score for flavor and odor, the free fatty acids of milk fat for all cows considered in this study were also determined. The acid degree determinations were limited to the samples of milk fat isolated for determination of carotene. Whereas the surface tension given here was measured on aging milk for 48 hours, the acid degree of milk fat was determined on samples aged 7 days. Before being churned for isolation of fat, the milk was heated to 75° C. for 5 minutes to prevent further lipolysis.

The acid degree of milk fat for cows 588 and 707 is presented in figure 6. As is readily seen, the data obtained by this method to measure the extent of hydrolytic rancidity agree with the data on surface tension.

SUMMARY

Six cows, 5 Jerseys and 1 Guernsey, were kept for various periods, extending up to 269 days, on a very low-carotene diet of bleached alfalfa hay and concentrates. On this diet the depletion of carotene in blood and milk fat was most rapid in the first 20 days; after about 40 days the carotene values of both the blood and milk fat leveled off at the values between 0.25 and 0.05 mg. of carotene per 100 cc. of blood plasma. The relation between the amount of carotene in blood and that in milk fat approached a factor of a direct proportionality when considered for each cow separately. The numerical values of carotene, moreover, are nearly the same in blood and in milk fat if expressed on the basis of mg. of carotene per 100 cc. of blood plasma and mg. of carotene per 100 cc. of milk fat.

With 4 cows, the depleted carotene was restored to normal by feeding either crystalline β-carotene (up to 1000 mg. daily, dissolved in cottonseed oil) or green alfalfa.

The data on the extent of lipolysis of fat in aged milk, measured by surface tension of milk and acid degree of fat, failed to reveal any consistent relation between the level of carotene in the blood or the milk fat of a cow and the extent of spontaneously developed hydrolytic rancidity in her milk.

This study has fully confirmed that the advanced stage of lactation is a contributing or major factor in the secretion of milk high in the concentration of a naturally active lipase. In this experiment every cow in a late stage of lactation and gestation on the diet of bleached alfalfa hay and concentrates secreted milk that spontaneously developed strong rancid flavor.

The limited data obtained in the present study support the observations made by other workers: the ingesting of green feed prevents the secretion of milk high in a concentration of a naturally active lipase and generally improves the flavor of milk.

A critical examination of the results obtained in this study warrants two conclusions: 1) that the principle responsible for the beneficial effect of green feed in preventing spontaneous lipolysis of the milk fat is not its high carotene content and 2) that the hydrolytic rancidity occurring in the milk of cows near the end of gestation on a dry-feed diet cannot be attributed to the low-carotene content of the ration.

CONCLUSION

The level of carotene in blood or milk fat is not *per se* the factor causing a cow to secrete milk that will spontaneously develop a hydrolytic rancidity.

ACKNOWLEDGMENT

The authors appreciate the assistance of Dr. L. J. Henderson in scoring the samples for flavor.

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HOMOGENIZING EFFICIENCY TEST STUDIES

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The following definition for homogenized milk appears in the United States Public Health Service Milk Ordinance and Code 1939 Edition: "Homogenized milk is milk which has been treated in such manner as to insure break-up of the fat globules to such an extent that after 48 hours storage no visible cream separation occurs on the milk and the fat percentage of the top 100 cc. of milk in a quart bottle, or of proportionate volumes in containers of other sizes, does not differ by more than 5 per cent[2] of itself from the fat percentage of the remaining milk as determined after thorough mixing."

It will be noted that the definition says nothing as to the temperature at which the sample shall be held in storage nor does it make it clear how a sample taken from a delivery vehicle shall be handled prior to testing. The definition does not specify the method of removing the sample of "top milk" from the bottle or the method to be used for butterfat determination.

ABILITY OF PLANTS TO COMPLY WITH U. S. PUBLIC HEALTH SERVICE HOMOGENIZING EFFICIENCY STANDARD OF 5 PER CENT

In presenting the following summary, it should be stated that a close check was kept on the plants involved and that probably better than average care was taken in an endeavor to meet the standard. The study covered the period of the first eleven months of 1942.

Fifty-two plants reported 6405 individual tests. They also reported these tests as a monthly arithmetic average for each plant based on a minimum of eight tests per plant per month. There follows certain factual information obtained by studying these data.

No. individual tests reported 6405.
No. tests over 5 per cent standard 970 or 15.14 per cent.
No. plant months involved (11 × 52) 572.
No. monthly averages over 5 per cent standard 85 or 14.9 per cent.
No. monthly averages over 5 per cent standard that fell between 5 per cent and 7 per cent—60 or 70 per cent of those over standard.

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[1] The writer wishes to give due credit for the work done by R. Whitaker and his staff at the Sealtest Research Laboratory, Baltimore, Maryland. Thanks are also due those in the various plant control laboratories for making various tests as suggested.

[2] Since this manuscript was prepared this figure has been changed to 10 per cent.

No. monthly averages over 7.1 per cent—25 or 30 per cent of those over the standard.

Twenty-nine of the 52 plants showed a monthly average test within the 5 per cent standard for all eleven months.

The difficulty in the case of 15 out of the 25 monthly averages of over 7.1 per cent was found to be old machines that had to be rebuilt or replaced. It would appear, therefore, that while the majority of plants might on the basis of monthly averages meet a 5 per cent standard, a 7 per cent standard would be more equitable and it is probable that a 10 per cent standard would be more reasonable.

Curd tension tests were also reported on the 6405 samples and it is of incidental interest to note the correlation between curd tension and per cent difference in fat between top and bottom tests in the two extreme groups. Thus, 153 monthly average differences of 2.5 per cent or less between top and bottom tests showed an average monthly curd tension average of 15.48 while 56 samples showing a top and bottom difference of 6 per cent or more showed a curd tension of 15.51. Evidently, homogenization can be quite inefficient before curd tension is materially affected.

During most of the year, the top 100 cc. of milk was pipetted from a quart bottle of milk after the sample had stood for 48 hours at a temperature of 40° to 50° F. The results obtained aroused our interest to know more about the factors affecting the efficiency of homogenization and to know what standard the plants could normally be expected to meet.

Through work in our Baltimore Research Laboratory, in the control laboratories in the field and by plant surveys, we have endeavored to study the factors that determine homogenizing efficiency and the accuracy of the test prescribed therefor. The factors involved in the sampling and testing technique will be discussed first.

A. TESTING PROCEDURE

1. *The Babcock vs. Mojonnier test*

Seventy-three comparisons were run using the two tests. The average per cent difference in the fat content of the top and bottom milk in the case of the Babcock test was 4.35 per cent and in the case of the Mojonnier test 4.14 per cent. This clearly indicates that it is not necessary to go to the extra expense of using the Mojonnier test to determine compliance with the homogenizing efficiency standard.

2. *Method of making the Babcock test*

It was found that the modified Babcock test the plants had used resulted in too many imperfect fat columns. The following procedure was developed and gives excellent results:

1. Warm sample to about 60° F.
2. Temper acid to 60° F. To 17.6 cc. milk add 15 cc. 1.82–1.83 sp. gr. H_2SO_4.
   Add in 3 or 4 portions.
   At time bottles are placed in centrifuge, no red tinge should be visible.
3. Centrifuge 10 minutes.
4. Add hot water to $\frac{1}{2}$–$\frac{3}{4}$″ below the neck.
5. Centrifuge 2 minutes.
   Shake before adding water.
   Add hot water to base of neck.
   Allow fat to coalesce and then fill up into neck.
6. Centrifuge 1 minute.
7. Read from water bath as usual.

3. *The influence of single vs. duplicate testing*

Table 1 presents the result of making five tests on the same top and bottom samples.

TABLE 1

The effect of running multiple tests on the same sample on the homogenizing efficiency test

	Butterfat				
Test No.	1	2	3	4	5
	%	%	%	%	%
Top	3.8	3.8	3.85	3.9	3.8
Bottom	3.6	3.6	3.6	3.65	3.6
% diff.	5.2	5.2	6.5	6.4	5.2

The data clearly show the effect of slight variations in reading the test, in this case varying from an average test difference of 5.2 per cent to 6.5 per cent. In another comparison, fifteen Mojonnier and twenty-five Babcock tests were run singly and in duplicate and the average difference between top and bottom test was 0.31 per cent greater when samples were run in duplicate and the results averaged. Duplicate testing is recommended. Only clear tests should be read and when duplicates differ by more than 1/10 of 1 per cent in the reading, the sample should be retested.

4. *Effect of personal factor in making the Babcock fat test*

It is, perhaps, natural that one's experience in making the Babcock test as well as one's carefulness might make some difference in the homogenizing efficiency test. In table 2 are shown the results of two or more operators on a series of tests.

It will be noted that in general different operators agree quite well but that for reasons mentioned above there can be, and occasionally is, quite a difference. Thus in the case of three of the series of tests, two different operators varied by as much as 2 per cent to 3 per cent in the final result.

The personal factor is another thing that has to be given some consideration in complying with the homogenizing efficiency test standard.

5. *Effect of possible error in reading the Babcock test (theoretical)*

The accuracy of the Babcock test is considered to be no greater than the smallest division on the test bottle. With the 8 per cent bottle now commonly used, the smallest division is 0.1 per cent. This means that on a sample of milk known to contain 4 per cent fat, for example, it might be possible to obtain tests of either 3.9 per cent or 4.1 per cent and still be

TABLE 2

The effect of the personal factor on the homogenizing efficiency test

No. tests	Operator No.				
	1	2	3	4	5
	Per cent difference between "top" and "bottom" tests				
6	1.8	1.1	.....	.....	.....
6	4.33	4.43	5.36	.....	.....
6	3.67	.....	4.75	.....	.....
6	6.02	.....	6.81	.....	.....
6	7.27	.....	8.61	.....	.....
6	2.23	5.07	.....	.....	.....
6	8.43	6.84	.....	.....	.....
6	11.05	9.22	.....	.....	.....
5	.....	.....	.....	5.7	6.1
5	.....	.....	.....	6.1	5.3

correct. These limitations of the Babcock test must be taken into consideration in considering the problem of complying with the U. S. Public Health Service standard. Assuming milk to be perfectly homogenized and the top test to be read 1/10 of a per cent too high and the bottom test to be read 1/10 of a per cent too low, it would be possible to have a difference of 2/10 of a per cent fat solely due to reading. If this condition prevailed, the per cent difference between top and bottom tests for milk with different fat content would be as is shown in table 3. These theoretical data show that it is easier to meet the standard when processing a high fat content milk.

TABLE 3

The effect of possible error in reading the test on milk of different fat content

3% fat milk % diff.	4% fat milk % diff.	5% fat milk % diff.
6.4	4.9	4.0

6. *Effect of fat content of milk*

The richer the milk, the less the per cent difference between the top and bottom test is likely to be. Thus two samples of milk testing 4.5 per cent fat showed a difference between the top and bottom test of 4.4 per cent whereas

two samples of milk testing 3.6 per cent fat showed a per cent difference between top and bottom test of 5.5 per cent.

7. *Method of removing top 100 cc. sample*

A study of various methods of removing the top 100 cc. sample of milk follows:

a. *Syphon.* A glass tube was inserted in a rubber stopper which was of such size that it just fitted into the neck of a quart milk bottle. The bottom of this tube was bent in the shape of a J, the outlet level being adjusted so that just 100 cc. of milk could be syphoned off. The other end of the tube was merely bent at an angle of about 45° to facilitate running the sample into a bottle or other vessel. The syphon was started by means of a slight air pressure applied under the stopper through another tube which was connected to a rubber bulb.

b. *Pipette.* The top 100 cc. of milk was removed by sucking the milk into a 100-cc. pipette, care being taken to keep the tip as near to the surface as possible without sucking air.

c. *Drainage.* A small hole was drilled in the bottom of several quart milk bottles and each was fitted with a rubber stopper carrying a short piece of glass tubing. A piece of rubber tubing and a pinch cock served to close

TABLE 4

The per cent difference between top and bottom tests when the top 100 cc. of milk from a quart bottle of homogenized milk is removed by various methods. (All samples stood 48 hours at 40° to 50° F.)

No. of samples	Top and bottom test differences			
	Syphon	Drainage	Pipette	Pouring
	%	%	%	%
5	4.97	8.9	7.28	10.44
323 by 16 plants or operators	.....	.....	3.63	4.56

the tube. When it was desired to take a sample, 846 cc. of milk was drawn off, leaving 100 cc. in the bottle. This also was drawn out after shaking the bottle, and represented the top 100 cc. layer.

In the case of paper bottles, a hole was made in the bottom of the container with an ice pick and 846 cc. allowed to run out. The hole was then closed and the remaining 100 cc. of top milk was shaken around in the bottle, and then drawn off.

d. *Pouring.* In this method the top layer of milk was merely poured off into a cylinder until a total of 100 cc. had been collected.

The above data showed definitely that results may be affected very decidedly by the method used for removing the top 100 cc. of milk. It is obvious that pipetting can be the most inaccurate of all because it is so easy for the operator to place the tip of the pipette a little below any "cream"

that may have risen to the top of the bottle. In this case, the results might easily show the standard to have been met whereas quite the reverse was true. The above data show that the pouring method is the most severe and hence gives the most nearly true results. The pouring method has certain advantages which made it the preferred method.

1) It is easy to conduct, the top 100 cc. merely being poured off the top of a regular milk bottle.

2) No special equipment is required, other than some container marked to hold 100 cc.

3) The results obtained with the pouring method are as high, if not higher, than the other methods tested, which is in its favor.

It was our thinking that the drainage method heretofore described would prove to be the most accurate method. Further work was done to determine why this was not the case and to further prove that the pouring method would be the one to adopt.

Into one of the drainage bottles, there was placed a quart of water. Fifteen cc. of water were removed and this replaced with 15 cc. of 5 per cent milk. This was carefully floated on the water so that two distinct layers were formed and no milk and water were permitted to mix. This simulated conditions which exist at the time the test is usually conducted. As the water was drained out, it was observed there was a mixing of the milk and water in the vicinity of the side walls of the bottle. As the liquid was lowered in the bottle, especially below the shoulder, a rolling action seemed to take place which caused the milk to stream down into the water, thereby reducing the fat in the top 100 cc. layer and increasing the fat in the lower or water layer.

In another bottle of water there was floated 15 cc. of 5 per cent milk as above, but in this case the milk was poured off in the manner usually employed. Examination of the top and bottom layers showed the lower water layer was virtually free of all but the slightest amount of milk.

From this demonstration it would seem that there is sufficient blending of the top and bottom layers during the time the drainage bottle is being drained to lower the fat test of the top layer and increase that of the lower layer. In the case of the pouring test, the milk layer seemed to flow off readily as the bottle was tipped, so that by the time the 100 cc. had been poured out, all of the milk layer had been removed. Although not exactly identical with the conditions existing with homogenized milk, it seems likely that this observation indicates the cause of the better separation which is noted when the sample is poured.

From the above studies it is concluded that the preferred method of removing the sample of top milk is the pouring method and this method was used in the tests reported in the balance of this paper.

8. *Effect of holding samples for 24- and 48-hour periods*

Forty-three laboratories cooperated in this test. Samples were held at a temperature of 40° F. to 50° F.

It will be noted that the average per cent difference between top and bottom tests is nearly doubled for 48 hours as compared to 24 hours. The number of samples failing to satisfy the standard is also greatly increased by the 48-hour holding period.

TABLE 5

The effect of the time of holding samples on the homogenizing efficiency test

No. plants	No. samples	% diff.	No. samples over 5%	No. plants with one or more samples over 5%
Samples held at 40° to 50° F. for 24 hours				
43	215	2.63	14	8
Samples held 48 hours				
43	320	4.5	117	35

9. *Effect of agitation of sample*

The following data indicate that a sample taken from a delivery vehicle or shipped to a laboratory and hence subject to an unknown amount of agitation as well as being of unknown age from time of bottling, should be inverted ten times and left to stand for 48 hours before testing. The lower per cent difference on the milk as returned from the route is due in part to the fact that 48 hours had not elapsed from time of bottling to time of testing.

TABLE 6

Effect of agitation on the homogenizing efficiency test

Trial No.	No. of samples	1 % diff.	2 % diff.	3 % diff.	4 % diff.
1	24	3.6	....	2.1	3.64
2	12	4.54	3.5	2.8	4.4

NOTE: In both trials sample 1 held undisturbed at 40° to 50° F. for 48 hours. Sample 3 held for 18 hours, sent out on route and tested as soon as returned to plant. Sample 2 held for 25 hours undisturbed at 40° to 50° F. Sample 4 inverted 5 times in trial No. 1 and 10 times in trial No. 2 after returning from route and then held undisturbed for 48 hours at 40° to 50° F.

10. *Effect of temperature at which samples are held for 48 hours*

That the temperature at which the bottle of milk is held for 48 hours is important is shown in table 7. It is difficult to specify a definite temperature. It is felt that a range of from 40° to 50° F. meets most situations and the data in the table indicate that there is not likely to be any marked difference in the test as between the temperature of 40° to 45° F. and 45° to 50° F.

As an indication of a problem that might arise in testing samples taken from delivery vehicles during very warm weather, some sets of samples were held at 47° F. for 48 hours and top and bottom tests made. The average per cent difference in fat between the top and bottom milk was 5.26 per cent in both cases. Duplicate samples were held for 24 hours at 47° F. and then placed in a 72° F. room and held for another 24 hours. One sample showed a top and bottom test difference of 7.5 per cent and another 11.4 per cent. It is pointed out that the difference between these two latter figures is due to the fact that the milk had expanded enough in one case so that some fat was adhering to the cap. It is evident that as the milk is warmed the fat rises to the top more easily. Furthermore if bottles are slightly overfilled, some of it is going to adhere to the cap and create a further problem.

TABLE 7

The effect of the temperature of storing the sample on the homogenizing efficiency test

No. samples	32° – 35° F. % diff.	40° – 45° F. % diff.	50° – 55° F. % diff.
32	3.15	6.07	8.73

No. samples	40° – 45° F. % diff.	45° – 50° F. % diff.
9	4.85	4.32

In checking the efficiency of homogenization, the question arises as to whether samples of warm milk taken from the outlet pipe of the homogenizer will check with samples of cooled bottled milk taken from the filler. There is a problem involved in cooling the warm milk samples taken from the hot milk line from the homogenizer.

Indications are that it takes so long to cool the quart bottle of milk taken from the hot line that the per cent difference between top and bottom tests at the end of 48 hours will be greater than on a bottle of milk taken from the filler at approximately the same time as the sample is taken from the hot line.

Thus, in one trial, a bottle taken from the filler showed a per cent difference between top and bottom fat tests of 4.6 per cent; a bottle taken from the hot line and cooled to 46° F. in ice water without agitation (time required—one hour), 8.4 per cent; a bottle allowed to cool to 46° F. in refrigerator (time required—seven hours), 11.3 per cent. In another similar test results were as follows: Bottle from the filler—8.4 per cent difference; bottle cooled in ice water—10.4 per cent; bottle cooled in air in the refrigerator—11.3 per cent.

11. *The size of the bottle*

While it is believed that the homogenizing efficiency test is generally made on a quart bottle of milk, it was deemed advisable to see whether equivalent results were obtained with pint and half-pint bottles, removing 50 cc.

of top milk in the case of the pint bottle and 25 cc. in the case of the half-pint bottle. Thirty sets of tests were run in all, using the same milk so that the results could be compared. The average top and bottom test difference in the case of the quart bottle was 6.13 per cent, for the pint bottle 6.94 per cent and for the half-pint bottle, 6.38 per cent. From this, we may certainly conclude that the U. S. Public Health Service standard applies equally as well to pint and half-pint bottles as to quart bottles.

12. *The glass vs. the paper bottle*

For some reason or other, cream does not rise as rapidly or as thoroughly in the paper bottle as in the glass bottle. Data to this effect have been previously published for unhomogenized milk. In all, 31 comparisons were made where the same milk was placed in both glass and paper bottles, the samples being held for 48 hours at a temperature of 40° to 50° F. The average per cent difference between top and bottom test in the case of the glass bottle was 7 per cent and in the case of the paper bottle, 3.84 per cent.

13. *Type of glass bottle used*

Glass bottles with neck diameter ranging from 46 to 56 millimeters are in use in the industry. Bottles of so-called "cream top" type are also in use. Samples from the same lot of milk were set aside in these different types of bottles and held for 48 hours. The results are shown in the following table showing the effect of the type of bottle on the homogenizing efficiency test.

TABLE 8

The effect of the type of bottle used on the homogenizing efficiency test

No. samples	46–48 mm. % diff.	56 mm. % diff.	56 mm. cream top % diff.
28	5.23	4.88	4.61 (20 samples)

From these data it may be concluded that the type of bottle used does not materially affect the Homogenizing Efficiency Test.

B. PROCESSING PROCEDURE

Regardless of the top and bottom test procedure, the real factors that affect the efficiency of homogenization have to do with the processing operation.

1. *The effect of the homogenizing pressure used*

This effect is set forth in table 9.

This table clearly shows that with homogenizers other than the Creamery Package Manufacturing Company's machine which is not designed to be used at such high pressure, a pressure of 3000 to 3500 lbs. is desirable for

good milk homogenization. Uniform, steady high pressure is of prime importance.

2. *Effect of time of sampling*

Eleven plants were asked to cooperate in this experiment. One bottle of homogenized milk was taken from the filler from among the first six bottles filled, another at about the middle of the run and a third from the last six bottles filled. The experiment was repeated on at least three different days. Results are reported on 35 sets of 3 samples each in table 10.

The results shown in table 10 indicate the possibility of relatively uniform test results throughout the run. No importance should be attached to the fact that the results appear to be slightly better as the run progresses.

TABLE 9

The effect of homogenizing pressure on the homogenizing efficiency test

No. samples	Pressure	% diff.
2	1500	22.15
2	2300	11.7
2	3000	6.3
2	3500	3.6

3. *Effect of homogenizing before and after pasteurization, i.e., holding*

Some plants homogenize before the holding period, others after the holding period, depending somewhat upon the wishes of the local Health Department in this respect. A series of six samples showed a top and bottom test difference of 5.73 per cent when homogenizing was done before the holding period and 3.7 per cent when done after the holding period.

TABLE 10

The degree of uniformity of the homogenizing efficiency test during different hours of operation

No. sets of samples	No. plants	1st of run % diff.	Middle of run % diff.	End of run % diff.
35	11	4.95	4.69	4.06

Incidentally, it may be well to mention at this point the results of bacterial counts on homogenized and unhomogenized milk of the same grade after processing in 49 plants. These averages were obtained by averaging the log average of approximately eight counts per plant. The average bacterial count for the homogenized milk was 7153 per cc. and for unhomogenized milk 9266 per cc. Slightly higher temperatures are generally used in pasteurizing the homogenized milk than the unhomogenized milk. The results certainly do not show any bacteriological contamination due to the homogenizing procedure.

4. *Effect of pasteurizing temperature—pressure constant*

Data on this point is presented in table 11.

The data clearly show that the higher the pasteurizing temperature, the smaller the difference between the test of the top and bottom milk. This presumably is due to the effect of heat tending to prevent the fat from rising. In one case, short time, high temperature pasteurization at 165° F. for 15 seconds was used. It is interesting to note that this gave essentially the same result as using 160° F. for 30 minutes.

TABLE 11

The effect of the pasteurizing temperature on the homogenizing efficiency test—pressure constant

145° F. – 30 min. % diff.	150° F. – 30 min. % diff.	155° F. – 30 min. % diff.	160° F. – 30 min. % diff.	165° F. – 15 sec. % diff.
6.68	5.55	3.7	2.6	3.1

5. *Effect of the homogenizing temperature—pasteurizing temperature constant*

Data on this point are shown in table 12.

The data in table 12 indicate that better results are obtained when the milk is homogenized at a temperature of 150° F. than at 120° F. The data further show that better results are obtained when homogenizing is done after the holding period.

TABLE 12

The effect of homogenizing temperature on the homogenizing efficiency test—pasteurizing temperature constant

Homo. 120° F. – Past. 150° F. % diff.	Homo. 150° F. – Past. 150° F. % diff.
6.2	4.4
Past. 150° F. – Homo. 120° F. % diff.	**Past. 150° F. – Homo. 150° F. % diff.**
4.9	3.1

6. *Influence of clarification*

In two different tests, milk clarified cold prior to processing showed an average difference between top and bottom test of 6.2 per cent. On two other occasions in the same plant where the milk was not clarified, the difference was 4.7 per cent. These data are, of course, rather meager and only serve to indicate that clarification may make it slightly more difficult to comply with the Homogenizing Efficiency Test Standard.

7. *Influence of use of impact ring in Manton-Gaulin homogenizer*

In the case of five tests, when the impact ring was used the average top and bottom test difference was 6.1 per cent whereas when the ring was not

used it was 8.1 per cent, indicating, of course, that better results may be expected when the impact ring is used with this machine.

8. *Condition of the homogenizer valves*

This is so well understood as to need no discussion.

9. *Imperfect gaskets*

Care must be taken to keep the gaskets between the homogenizing valve chamber and the homogenizing valve in good condition. If these gaskets become scored or distorted, unhomogenized or poorly homogenized milk can pass through such imperfections in the gasket and mix with the properly homogenized milk on the other side of the valve.

10. *The use of clean equipment*

Homogenized milk should be the first product processed after a clean-up. If homogenized milk is handled through equipment following unhomogenized milk or cream, it will be contaminated with unprocessed fat. If used after some other product, the lines and equipment should be thoroughly rinsed to wash out all unprocessed fat. It is recommended that a by-pass arrangement be installed on the homogenizer which will direct the first milk through the machine at the start or following shut-downs back into the unhomogenized supply. If the homogenized and cooled milk can run into a large surge tank and be gently agitated before bottling, that will help.

11. *Amount of air in milk*

If air is sucked into the intake line and passed through the homogenizer with the milk, poor homogenization results. The intake line should preferably be under a slight positive pressure and the homogenizer never allowed to run out of milk. Where it is impossible to pump the milk to the homogenizer, great care must be taken to see that there are no leaky unions or connections in the suction line. Also, great care must be taken to see to it that the opening of the supply tank is always covered. When a machine sucks air, the pressure gauge indicator will oscillate badly and this always means poor homogenization.

It will be seen from the above that if milk is to be properly homogenized, it is a process that must be carefully watched. It must have the constant attention of an operator throughout the run.

METHOD OF INVESTIGATING CAUSES OF FAILURE TO MEET THE HOMOGENIZING EFFICIENCY STANDARD

A questionnaire asking every conceivable question that we could think of that might have a bearing on the results of the homogenizing efficiency test was sent to more than fifty milk plants. The results of the survey for a number of the plants have been tabulated. The answers were arranged

in two tables; those coming from plants with an average top and bottom test difference of 5½ per cent or more in one table and those coming from plants with an average test difference of 3½ per cent or less in another table. To our surprise and disappointment, it was found that after studying the data in these two tables, one could not hope to get the answer as to why one plant was having difficulty in meeting the standard while another was meeting the standard without difficulty. Apparently the only way the answer to unsatisfactory compliance can be obtained is for a properly trained person to visit the plant and study the operation for a considerable period of time. I say this because when the answers to our questionnaire were arranged in the two tables just mentioned, there appeared to be an equal number of reasons in both tables as to why a plant would or would not be expected to meet the standard.

SUMMARY

1. With very careful control procedures, a plant should be able to meet the U. S. Public Health Service homogenizing efficiency test most of the time. It is felt, however, that a more liberal test would be more practical since there appears to be no reason for such a rigid test.

2. The Babcock test as per modification presented is the practical test to use in determining homogenizing efficiency.

3. All fat tests should be run in duplicate and if duplicates differ by more than 1/10 of 1 per cent, a retest should be made. Tests showing fat columns that are not perfectly clear should be discarded and retests made.

4. Persons well trained in the making of the Babcock fat test will differ but little in the results obtained when making the homogenizing efficiency test.

5. The theoretical possible error of 2/10 of 1 per cent in reading top and bottom tests may easily be the determining factor as to whether the homogenizing efficiency test standard is complied with.

6. The richer the milk, the less the per cent difference between the top and bottom test is likely to be.

7. The pouring method is the preferred method for removing the top milk from the bottle in making the homogenizing efficiency test.

8. The top and bottom test difference at the end of 24 hours' storage of the sample is approximately one-half that obtained when the sample is held for 48 hours.

9. Samples taken from routes or shipped to laboratories for testing should be inverted ten times and held for 48 hours at 40° to 50° F. before testing.

10. If the sample is held at a temperature below 40° F. the difference between the top and bottom test is much less than when the sample is held at temperatures above 50° F. There appears to be no significant difference

if the sample is held within a range of 40° F. to 50° F. which it is concluded is a practical range for the average plant.

11. The homogenizing efficiency test works equally well on quart, pint and half-pint bottles.

12. The difference between the top and bottom test in milk held in a glass bottle is much greater than where the milk is held in a paper bottle for the same length of time.

13. Essentially the same results are obtained in bottles having a different top diameter and bottles of so-called "Cream Top" style.

14. High and steady homogenizing pressure is essential to good results.

15. The difference between the top and bottom test tended to be less when homogenization followed pasteurization.

16. The higher the pasteurizing and homogenizing temperature, the less will be the difference between the top and bottom tests.

17. It is possible that clarification may make it more difficult to meet the homogenizing efficiency test.

18. Failure to use the impact ring in the Manton-Gaulin Homogenizer makes it more difficult to meet the homogenizing efficiency test.

19. Other factors making it more difficult to meet the homogenizing efficiency test are:

(a) homogenizer valves in poor condition.

(b) imperfect gaskets between the homogenizing valve chamber and the homogenizing valve.

(c) the use of the processing equipment for both homogenized and unhomogenized milk.

(d) the incorporation of an undue amount of air in the milk prior to homogenizing.

20. Homogenizing can be quite inefficient as determined by the U. S. Public Health test before the curd tension of the homogenized milk is appreciably affected.

21. The bacterial count in homogenized milk need be no higher than in unhomogenized milk. Our results showed it to be lower, possibly due to the fact that higher temperatures were generally involved in processing the homogenized milk.

A METHOD OF EQUALIZED FEEDING FOR STUDIES WITH DAIRY COWS

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INTRODUCTION

Among the many problems of experimental design, there are some which deal with the elimination of bias from the main effects in which the investigator is interested, and some which deal with the minimization of the unaccounted-for variability of the experimental group (error of experiment) so that precise comparisons may be made. Published studies concerning the experimental methods which are used in feeding trials with dairy cattle have dealt primarily with the control of production ability and non-dietary environmental factors, especially as these variables change with respect to time. Along these lines Maynard and Myers (3) have published valuable suggestions for the improvement of continuous trials, and Cochran *et al.* (1) have presented designs which allow proper comparisons of two to four rations by means of change-over trials. Critical thought has apparently not been given to the control of dietary variables other than those under study.

Common to almost all dairy cow experiments is the practice of initially basing the rate of concentrate intake of each individual upon the rate of milk and fat production of that individual, and thereafter periodically adjusting the rate of concentrate intake to the immediately preceding rate of milk and fat production. In some experiments roughage feeds are allowed *ad libitum*, but under these conditions it is difficult to evaluate the production efficiencies of rations because of unknown intakes or of the possibility of variable roughage consumption. To overcome this difficulty, therefore, many experiments are carried out under conditions of arbitrarily controlled roughage feeding. The rates of roughage feeding for each individual are determined by the investigator, primarily on the basis of body weight, but with consideration given to individual peculiarities such as age and condition. The rates are usually maintained constant for each animal throughout the experiment.

When both concentrate and roughage intakes are controlled during a comparison, either production will fall on the poorer ration or, if production is maintained for a time, body tissue will be sacrificed. In either case, production will suffer sooner or later. When employing the usual method of periodically adjusting concentrate allowances to production, the concentrate intake will eventually become lower on the inferior ration than on the better ration. Thus, the animals on the less efficient ration will be subjected to two penalities, first, an inferior ration, and second, a lower total nutrient

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intake. When employing the continuous trial, the differences in production values of rations may, therefore, be magnified. In the change-over trial, however, because of the reversal of rations, the effects of shifting nutrient intakes will tend to reduce the real difference between rations. In both types of trials the magnitude of differences observed may be rather inconsistent among experiments, because of varying availability of body tissue.

In case the change in rations at the start of an experimental period produces a temporary effect on production, then this temporary effect may be prolonged because of the changes in total nutrient intake which result from adjusting the grain intake to the immediately preceding production. Even though recovery to normal does occur on a temporarily poorer ration, it can only occur at the expense of body tissue. Thus, recovery may not always occur, and a temporary difference might appear as a permanent one.

A study of experimental groups of cows, under conditions where both concentrate and roughage intakes were controlled, suggested that the greater portion of the unaccounted-for variability was associated with differences among individuals as regards decline in production rate (persistency). Certain considerations warranted the conclusion that a portion of this variability in persistency was attributable to the methods used for initially determining and periodically adjusting total nutrient intakes.

As was previously pointed out, total nutrient requirements and intakes are based upon body weight and the rate of milk and fat production. It is obvious that, regardless of the skill of the investigator in adjusting to individual peculiarities, all animals of a group can not be fed at the same nutrient level relative to their actual requirements for maximum production at a given time. Compared to the average rate of feeding for the group, varying degrees of overfeeding and underfeeding will occur. Consider that a particular animal is underfed. Her production will eventually fall to a level compatible with her inadequate nutrient intake. When the decrease in production occurs, concentrate intake will then be reduced, so that the particular animal will be even more greatly underfed relative to the average rate of feeding for the group. A further drop in production will follow. Since this process is continually repeated, a lower than average persistency will ensue. On the other hand, an animal which is overfed relative to the average for the group will show a higher than average persistency. The more often adjustments of concentrate allowances are made, the greater will be the effects of small errors in the initial assessment of nutrient requirements.

As has been discussed, the usual method of controlling nutrient intake may distort a permanent difference between rations or prolong temporary effects. In other words, a bias is introduced which will always favor the better rations during comparisons by the continuous method and the poorer rations during changeover trials. To overcome this bias some investigators

have used paired feeding, yet no critical reports upon the efficiency of this method for dairy cattle studies have been published. This method has been of great value in growth studies with other animals, but certain considerations limit the application of this technic to dairy cattle experimentation.

In growth studies any change in energy stores (in excess of essential tissues) is reflected in body weight which is used as the measure of growth. Thus, the criterion of comparison (body weight) is always highly dependent upon total energy intake and the efficiency of the ration, and when two animals of the same initial weight and age are fed identical energy intakes, the unaccounted-for variability will automatically be limited, allowing precise comparisons. In the case of lactating animals, however, the criterion of comparison (milk and fat production) may be substantially independent of total energy intake and the efficiency of the ration, at least for a time, because body stores may be increased or decreased while a relatively constant production rate is maintained. Therefore, if two animals are to be fed identical energy intakes, several criteria of pairing must be used, namely, body weight, milk and fat production, age and condition. If these four criteria are not all used, various adjustments to the set nutrient intake will affect production and thereby color comparisons or introduce variability sufficient to mask effects. It is difficult to obtain sufficient numbers of pairs for precise experiments when these four criteria are simultaneously utilized. Indeed, it is difficult to obtain sufficient numbers of pairs even from the standpoint of milk and fat production, because the populations from which experimental subjects may be chosen are usually limited and extremely variable. The investigator is then confronted by the problem of whether he should select a limited number of good pairs, or a large number of less desirable pairs. In the first case, the numbers of pairs will not be sufficient for precise comparisons. In the second case, a high unaccounted-for variability will limit precision. In either case, the technic is of application only when rather large differences are expected between rations.

EQUALIZED FEEDING

In order to make comparisons of rations for milk production more valid, the biases introduced by unequal changes in nutrient intake must be eliminated. To obtain more precise comparisons, the effects on production, attributable to errors in estimation of requirements must be compensated for so that more uniform persistency is allowed among animals. A method of determining and adjusting nutrient intake, hereafter referred to as equalized feeding, was developed which incorporated these two necessities. The method may be applied in conjunction with either the continuous type of trial when set up according to the specifications of Maynard and Myers (3), or the change-over type of trial when designed after Cochran *et al.* (1). There is a minor difference in technic for the two types of trials.

In applying equalized feeding the total time of experimental observation is divided into three parts, a preliminary period, a standardization period and a comparison period. The preliminary period, during which all animals are fed the same ration, allows an estimation to be made of the requirements of the animals, considering production, body weight, condition, age and appetite. The standardization period serves two purposes. First, it allows the animals to adjust to the errors made in estimating requirements during the preliminary period. Second, it allows the animals to stabilize at the adjusted production levels. This is of great importance in the continuous trial, because productions during the standardization period must serve as reference points for the determination of ration effects. The comparison period serves an obvious purpose, since during this time the experimental rations are fed. When using change-over designs, the comparison period is divided into a number of subperiods equal to the number of rations being compared.

Throughout the preliminary period each animal is individually fed and considerable care is necessary in adjusting feed intakes to individual peculiarities. It is best to first consider the rate of grain feeding which it is desired to maintain during the comparison period, and then to supply roughage at such rates that each animal holds essentially constant in her production when all animals are receiving approximately the correct amount of concentrates per unit of milk produced. This may require several adjustments of roughage supply. The preliminary period should begin about the time all animals have reached their peaks of production and are well on feed. The length of the preliminary period is determined by the length of time required to establish concentrate and roughage feeding at rates compatible with normal behavior of the animals. This will probably require two to four weeks.

Equalized feeding is begun the second week of the standardization period. It is continued throughout the remainder of the standardization period and the entire comparison period. Essentially, equalized feeding consists of periodically changing the daily concentrate allowances of all animals on all rations exactly the same percentage of their respective initial daily concentrate intakes. Meanwhile, the rate of roughage feeding is maintained constant for each particular animal at the level found to be adequate during the latter part of the preliminary period. The initial daily concentrate intakes are considered as those fed during the first week of the standardization period. They are based on the productions of each cow during the last week of the preliminary period.

The amounts by which the concentrate intakes are to be periodically changed might be determined arbitrarily by the investigator. For example, at the beginning of each week, the daily concentrate allowances might be reduced to 98 per cent of those furnished during the previous week. It is

customary, however, to keep concentrate intake at all times consistent with production level. If this is desired, then the changes in concentrate intakes may be based upon the average production behavior of all cows regardless of the rations which they are receiving. When this procedure is followed it is necessary to consider, in addition to the initial concentrate intakes, the initial levels of production as well, that is, production during the latter part of the preliminary period. To determine the concentrate intakes for any given week by this method, the following calculations are necessary. The sum of productions of all cows during the week previous to the week in question is divided by the sum of productions of all cows during the final week of the preliminary period. This yields a group persistency factor. The initial daily concentrate intake of each animal is then multiplied by this factor to obtain the daily concentrate allowance of each animal for the particular week desired. These calculations are illustrated briefly in table 1, productions during each week being expressed as average daily pounds

TABLE 1

A brief illustration of the calculations involved in equalized feeding when the concentrate allowances are adjusted once each week to the average production behavior of all cows regardless of ration

Cow no.	Last week of preliminary period	Periods of equalized feeding				
		First week		Second week		Third week
	Initial FCM.	Conc. TDN. equals initial FCM. times 0.324*	FCM.	Conc. TDN. equals 1st wk. TDN. times 0.977†	FCM.	Conc. TDN. equals 1st wk. TDN. times 0.959‡
1	60.0	19.4	58.5	19.0	57.0	18.6
2	52.0	16.8	51.0	16.4	50.0	16.0
3	45.0	14.6	44.0	14.2	43.5	14.0
	157.0		153.5		150.5	

* 0.324 equals pound TDN. per pound FCM. as recommended by Morrison (4).
† 0.977 equals sum of 1st wk. FCM. divided by sum of initial FCM.
‡ 0.959 equals sum of 2nd wk. FCM. divided by sum of initial FCM.

of four-per-cent fat-corrected milk (FCM), and concentrate intakes being expressed as pounds of total digestible nutrients per day (Conc. TDN). Instead of expressing the concentrate allowances in terms of total digestible nutrients they might be expressed on the basis of actual pounds of concentrates fed. The particular measure used would depend upon the aims of the investigation. The calculations illustrated in table 1 are continued during the standardization and comparison periods, always using the initial concentrate intakes and initial productions as bases for the calculations.

In ordinary experimental herds it is desirable for economic reasons to keep production as high as possible without invalidating experimental results. If one of the rations being compared were markedly inferior in pro-

duction value then the method of basing changes in concentrate intake upon the average behavior of all cows regardless of ration would result in an appreciable lowering of production in the entire experimental group. In such a case it would seem advisable to base the changes in concentrate intake solely upon the average behavior of the animals on the better ration. The persistency factor would be calculated in exactly the same manner as previously, using exclusively, however, the productions of those cows on the better ration. This factor would then be used to calculate concentrate allowances for cows not only on the better ration, but on the poorer ration as well.

It is seen that equalized feeding may be applied in several ways, namely, the changes in concentrate allowances may be determined arbitrarily by the investigator, they may be determined by the average behavior of all cows on an experiment regardless of ration received, or they may be based upon the average behavior of the cows on one particular experimental ration. Although the three ramifications of equalized feeding may result in altogether different absolute productions on the diets being compared, the observed relative differences between diets should be almost identical by the three methods when studying ordinarily used good diets. This is because in all cases the changes of concentrate intakes of all animals regardless of ration have been maintained equal at all times. It cannot be stated that the differences will be absolutely identical by the three methods, because theoretically there are possible interactions between ration quality and level of nutrient intake. Little is known concerning these interactions. Experience would indicate, however, that under usual conditions they are of a magnitude definitely insignificant compared to experimental error, even in refined experiments.

In the continuous trial, ration effects can be determined only by comparing the difference in production rate between the standardization period and the comparison period for one treatment group, with the difference between the two periods for another treatment group. That is, by determining the treatment-group by period interaction. In the continuous trial, therefore, the standardization and comparison periods are of equal importance. Because of this fact, the standardization period must be of sufficient length, not only so that adjustments to feed allowances are complete, but, in addition, so that the adjusted productions are maintained sufficiently long by all animals to ensure a reliable estimation of relative production abilities. During this time all animals are fed the same ration. The standardization period should be continued five to seven weeks, depending upon the degrees of overfeeding and underfeeding which are represented among the various animals when the standardization period is initiated.

Before the comparison period is begun, statistical tests may be used to discern the adequacy of standardization. In applying statistical tests for

this purpose, analysis of variance should be used. The criterion of adequacy is the week by cow interaction or variance which is not accounted for by the differences between week means and differences between individual cow means. For precise comparisons, this variance, translated to standard error of a single observation, should not exceed 2.5 per cent of the mean production rate of all cows for all weeks included in the analysis. When standardization is adequate the animals should be allotted to experimental rations and the comparison period begun. The first week or two of the standardization period, where the major adjustment fluctuations will occur, should be omitted for the analysis of adequacy of standardization. In order that the average productions during the standardization period may serve as reliable reference points for ration effects, the first one or two weeks should likewise be omitted in interpreting results.

In the case of change-over trials, the standardization period need not be more than two or three weeks in length, since adequate standardization of the animals is obtained during the comparison period by virtue of the rotation of rations. It is necessary, however, to utilize this short standardization period in order that the major fluctuations in production due to the adjustment of the animals to their particular nutrient intakes shall be practically complete. Unless this is done, the fluctuations of adjustment will color experimental comparisons. It was pointed out that in the continuous trial, all animals are fed the same ration during the standardization period, and are not allotted to experimental rations until the end of the standardization period. In the change-over trial, however, the animals are allotted to different rations at the start of the standardization period. If it is desired to estimate carry-over effects according to the method presented by Cochran *et al.* (1), the animals should be allotted to the same ration at the start of the standardization period as they are to receive during the first comparison sub-period. This is because the method presented by Cochran *et al.* assumes that no carry-over effects are present in the first comparison sub-period. On the other hand, if it is desired to study the transition effects immediately following a change in ration, the animals should be allotted to the same ration at the start of the standardization period as they are to receive during the last comparison sub-period.

When using equalized feeding in conjunction with change-over trials, both concentrate and roughage intakes will, by virtue of the rotation of rations, be automatically equalized on all rations when the trial is completed. This is not the case, however, in the continuous trial. Slight discrepancies in concentrate and roughage intakes will always be present among groups. It should be pointed out, however, that these discrepancies will be of equal magnitude during the standardization period and the comparison period, therefore, they will not significantly color ration comparisons when the data is properly analyzed. It is improbable that any possible bias introduced

by the interactions of ration quality with the slight discrepancies in concentrate and roughage intake would approach significance when compared with the error of experiment.

EFFICACY OF THE METHOD

It needs no illustration that equalized feeding removes a bias from ration comparisons. That equalized feeding allows a more uniform persistency among animals, thus increasing precision, is shown in the accompanying figures. There are compared two groups of twelve animals each, one group fed according to the usual method (individual feeding) and the other according to the equalized method. The data are presented on arithlog scale as production rates plotted against time. In figure 1 the results are shown as they would appear during a change-over trial with four five-week com-

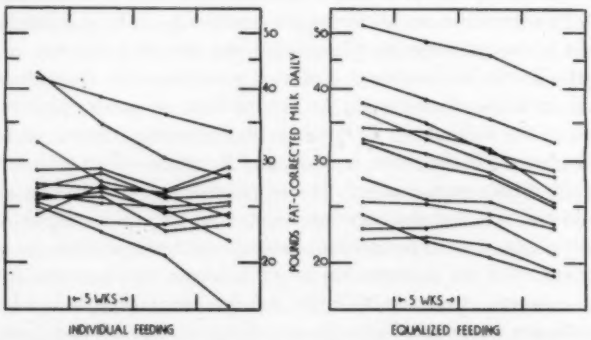

FIG. 1. A comparison of the uniformities of persistency within a group of cows fed according to the usual method (individual feeding) and within a group which was equalized fed. Data presented as they would appear during a change-over trial with four five-week comparison sub-periods.

parison sub-periods. Note how nearly parallel are the individual curves of the group maintained under equalized feeding. This is in contrast to the wide variation of the slopes of the curves of the group fed according to the usual method. In figure 2 the data are presented as they would appear for a continuous trial with a five-week standardization period and a fifteen-week comparison period. The uniformity of persistency during equalized feeding is again very striking.

Equalized feeding was used at this station in conjunction with a change-over trial which has been published elsewhere (2). The experiment was found to be extremely sensitive. In this trial there were studied two levels of fat intake and two levels of intake of the fat-soluble vitamins. In effect, four rations were compared. These were studied using the Latin square design for twelve cows as indicated by Cochran *et al.* (1), with four five-

week periods. Each Latin square consisted of 4 animals and represented different producing abilities (production levels), namely, high, medium and low producers. The summary table of the analysis of variance for fat-corrected milk production in this experiment is shown in table 2. It is seen that the unaccounted-for variance of the experimental group (residual period by animal interaction) was extremely low. The error terms for the various effects were quite constant and around 0.40. Taking the square root of this value yields 0.63 pounds as the standard error of a single observation. Since the mean production was about 28 pounds of fat-corrected milk per day, the standard error of a single observation was only 2.2 per cent of the mean. Usually the standard error of a single observation in lactation studies is 5 per cent or greater. The use of equalized feeding was, therefore, quite effective in increasing precision. In this particular experi-

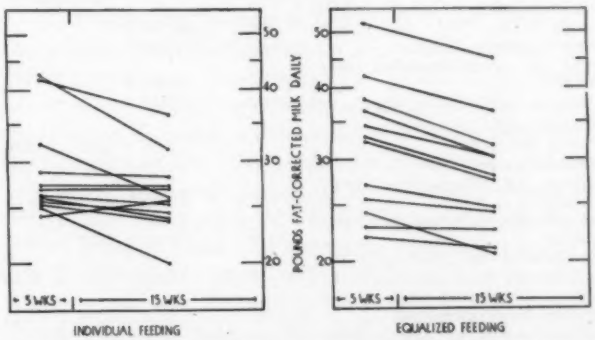

FIG. 2. A comparison of the uniformities of persistency within a group of cows fed according to the usual method (individual feeding) and within a group which was equalized fed. Data presented as they would appear during a continuous trial with a five-week standardization period and a fifteen-week comparison period.

ment where 24 observations were made on each fat level and on each vitamin level, the least difference between fat levels or vitamin levels necessary for high significance was of the order of 1.8 per cent.

Calculations show that to demonstrate a given difference as significant it would require approximately one-fourth as many animals when using equalized feeding as when using the ordinary method. Conservative estimates indicate that with equalized feeding and 20 animals, differences between rations as small as 3.5 to 5.0 per cent could be shown as significant in the continuous trial, and differences of 2.0 to 3.0 per cent in the change-over trial.

It is commonly considered that dairy cattle may be used for experimental purposes until about the twentieth week of pregnancy. Under conditions of equalized feeding, however, production rates fell off distinctly, as early

as the eighteenth week after conception in the case of medium and high producing cows. Thus, to ensure precision and lack of bias when making comparisons, it would appear advisable to use no animal which would have passed the eighteenth week of pregnancy at the close of the comparison period. This may require that the breeding of the animals which freshen first be delayed a few weeks.

TABLE 2

Analysis of variance of a change-over trial conducted with equalized feeding

Classification of variance	Degrees freedom	Sums of squares	Variance	Ratio (F value)	Significance*
Preliminary analysis:					
(1) Total	47	2662.01	.....	.....	.....
(2) Between production levels	2	1609.21	.....	.....	.....
(3) Between animals within production levels	9	631.16	.....	.....	.....
(4) Between periods	3	387.13	.....	.....	.....
(5) Period by production-level interaction	6	19.49	.....	.....	.....
(6) Period by animal interaction	27	15.02	.....	.....	.....
Analysis of dietary effects:					
(7) Between fat levels	1	5.95	5.95	15.26	SS
(8) Between vitamin levels	1	0.15	0.15	0.42	ns
(9) Fat-level by vitamin-level interaction	1	0.39	0.39	1.00	ns
(10) Fat-level by production-level interaction	2	0.65	0.32	0.82	ns
(11) Vitamin-level by production-level interaction	2	0.02	0.01	0.03	ns
(12) Fat-level by vitamin-level by production-level interaction	2	2.12	1.06	3.21	ns
(13) Residual period by animal interaction	18	5.74	.....	.....	.....
Errors to test for significance of dietary effects:†					
Between fat levels (9+10+12+13)	23	8.90	0.39	.....	.....
Between vitamin levels (9+11+12+13)	23	8.27	0.36	.....	.....
First order interactions (12+13)	20	7.86	0.39	.....	.....
Second order interaction (13)	18	5.74	0.32	.....	.....

* SS = highly significant. S = significant. ns = not significant.

† In the actual experiment 4 degrees of freedom were dropped from all error terms to compensate for 4 substituted values. These substitutions were warranted by an "off feed" during one period with one cow and effects of pregnancy during the last three weeks of the experiment with 3 other cows. This made the experiment slightly less sensitive than indicated here. Error variances were around 0.50.

SCOPE OF APPLICATION

When the differences between rations involve rather large effects on appetite, there is no point in using equalized feeding. The method would appear, however, to have a particular place as an aid in evaluating the small yet economically important differences which possibly exist among the multitude of rations classed today as good rations. Identical procedures would

be used whether it was desired to study the concentrate or roughage components of the ration.

The method would also appear to have a useful place in studies which are more fundamental than feeding trials. It is often not only desirable, but necessary to have a high degree of control upon energy intake in physiological and biochemical studies.

SUMMARY

The difficulties encountered in dairy cow experiments when using the usual method of periodically adjusting feed intake to production have been discussed. It has been pointed out that the method introduces a bias into ration comparisons, and that under these conditions there is a high variability among experimental animals as regards persistency. The latter effect reduces the precision of experimental comparisons.

A method of equalized feeding is presented, which prevents biases in ration comparisons, and simultaneously increases precision by allowing more uniform persistency among animals. Essentially, the method of equalized feeding consists of periodically changing the concentrate intakes of all animals on an experiment regardless of ration, exactly the same percentage of their respective initial concentrate intakes. Meanwhile the roughage portion of the ration is maintained constant for each animal.

The method may be applied in conjunction with either the continuous or change-over types of trials. When using equalized feeding and about twenty animals, it would be expected that differences of 3.5 to 5.0 per cent could be demonstrated as significant in the continuous trial, and differences of 2.0 to 3.0 per cent in the change-over trial.

Under conditions of equalized feeding, depressing effects on production rates were noticed as early as the eighteenth week of pregnancy.

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CERTAIN DIETARY FACTORS ESSENTIAL FOR THE GROWING CALF

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The observation by Phillips *et al.* (5) that nutritional scours in calves could be effectively prevented and cured by supplying adequate vitamin A and certain members of the "B" complex has led to a study of certain aspects of the nutrition in the very young calf. With the exception of research dealing with the role of antibodies and attempts to successfully raise calves without colostrum, very little work has been done to establish the dietary essentials needed by the newborn calf and to correlate the lack of specific dietary factors with calfhood diseases. With this in mind a series of experiments have been made to determine the dietary essentials necessary for the calf in its early life.

METHODS

The approach to this problem was to ascertain the nutritional status of the calf at birth and to follow it for an interval of six weeks. Blood analyses for vitamins A, C, and carotene were made on newly dropped calves before nursing occurred. The freshly drawn blood samples were immediately placed in an iced thermos jug for chilling and to avoid light. Vitamin A and carotene were determined by Kimball's method (1). All vitamin C determinations were made by the method of Mindlin and Butler (4).

Because of the importance of fat and antibodies of colostrum milk in the diet of the calf, attempts were made to attack this problem by means of a skimmilk diet from birth without allowing the calf access to colostrum. Unless otherwise indicated all results were obtained with calves fed a basal diet consisting of only skimmilk with a grain mixture and alfalfa hay accessible after the tenth day. Using this diet, experiments were then inaugurated to determine: 1) the effect of a vitamin A or carotene supplement; 2) the effect of ascorbic acid; 3) the effect of the vitamin B complex supplements; or 4) the effects of various combinations of these factors.

RESULTS

The blood picture of the newborn calf. It is evident in table 1 that the blood level of vitamin C in the newborn is considerably above normal for older calves. This level dropped rapidly in the first 24 hours and continued to drop at a somewhat slower rate to reach its lowest point at about two weeks. A rise in the blood plasma C then occurred which leveled off close to 0.30 mg. per 100 cc. of plasma.

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The blood plasma vitamin A and carotene were extremely low at birth. This observation supports previous work in this laboratory (5) and the work of Krauss *et al.* (3) in which they found vitamin A and carotene to be very low in the liver. Normal blood concentrations of 0.15 to 0.18 micrograms per cc. were reached within two days. The blood plasma carotene follows

TABLE 1

The blood concentrations of certain vitamins in the newborn calf

	Birth	1 day	2 days	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
Number of cases	45	17	13	21	9	12	10	8	9
Blood plasma C mg./100 cc.	0.70	0.38	0.31	0.30	0.19	0.27	0.25	0.31	0.30
Blood plasma A µg./cc.	0.05	0.14	0.18	0.16	0.15	0.17	0.12	0.16	0.17
Blood plasma carotene µg./cc.	0.05	0.31	0.36	0.31	0.29	0.17	0.16	0.15	0.14

the same trend as that of the vitamin A. The carotene levels are about twice those of the vitamin A during the first two weeks, thereafter under the conditions used in this study an abrupt drop occurred which reached a level of 0.14 micrograms per cc. by the end of six weeks.

TABLE 2

Blood plasma levels of ascorbic acid in calves raised on skimmilk supplemented with shark liver oil

Breed	Calf number	Birth	1 day	2 days	3 days	5 days	7 days
		Ascorbic acid, mg. per 100 cc. blood plasma					
Holstein	4	1.12	0.57	0.28	.....	.....	0.10
Jersey	706	0.46	0.20	.....	0.06†	.....	.....
Brown Swiss	834	0.46	0.09	.....	0.03†	.....	.....
Holstein	225*	0.82	0.30	.....	0.24	0.00	0.09
		10 days	14 days	21 days	28 days	35 days	42 days
		Ascorbic acid, mg. per 100 cc. blood plasma					
Holstein	4	.....	0.03	0.14	0.06	0.19	0.24
Jersey	706	.....	.....	.....	.....	.....	.....
Brown Swiss	834	.....	.....	.....	.....	.....	.....
Holstein	225*	0.04	0.00†	.....	.....	.....	.....

* Carotene fed as sole source of vitamin A.
† Died.

The effect of vitamin A or carotene supplement. Because of the low vitamin A concentrations in the blood of the newborn calf the data obtained in table 1 suggested that vitamin A was probably the principal vitamin supplement required, since 500–700 units of vitamin D daily, were supplied by

feeding irradiated ergosterol. That this was not true, however, was indicated by the fact that two of the first three calves fed skimmilk supplemented with shark liver oil, as a source of vitamin A, died from peritonitis on the fourth day. Since the initial concentrations of ascorbic acid in the blood of the calf are high and since the only calf to survive had an abnormally high blood plasma ascorbic acid level at birth, the data in table 2 suggested that ascorbic acid might prove beneficial. In the succeeding experiments vitamin C and the B-complex were given. Adequate blood plasma vitamin A was maintained by feeding shark oil so that the calf received 6,000 micrograms vitamin A daily for the first two or three days, 3,000 micrograms daily for another seven days, and 1,500 micrograms daily thereafter. The blood

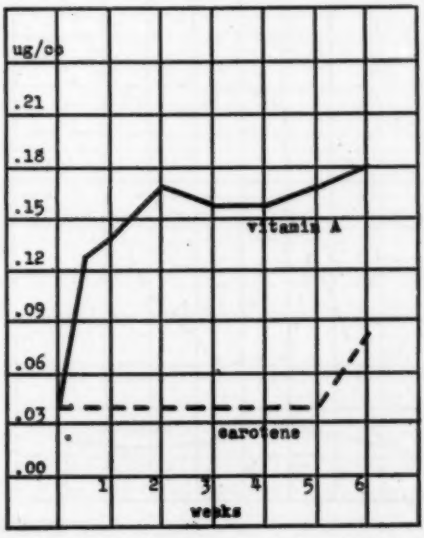

FIG. 1. Average vitamin A and carotene levels in the blood of ten Holstein calves fed shark liver oil and skimmilk.

plasma vitamin A and carotene under this regimen are given in figure 1. The blood concentrations were well within the range of normal calves for vitamin A.

A preliminary experiment with a new born Holstein calf indicated that it could not use carotene as the precursor of vitamin A. This is indicated in figure 2. The calf was fed skimmilk from birth plus additions of pure beta carotene in sesame oil (330,000 micrograms daily for seven days and 165,000 micrograms thereafter). For the first eight days this oil was hand mixed into the milk and thereafter by a small homogenizer. This apparently accounts for the rapid rise in blood plasma carotene after the eighth

day. This calf died on the 15th day. It showed typical symptoms of night blindness from birth. On autopsy the left eye appeared cloudy and the optic foramen was noticeably restricted. The abomasum, intestines, bladder, abdominal cavity, and heart were hyperemic and the calf was on the verge of peritonitis. Because of the disastrous results with this calf and scarcity of experimental calves the experiment has not yet been repeated.

The effect of additions of ascorbic acid. Since the evidence suggested the additions of vitamin C, it was given per os at the rate of 0.25 to 0.50 grams daily for varying periods up to 20 days. This addition eliminated the loss of calves from peritonitis. Evidence was obtained that the feeding

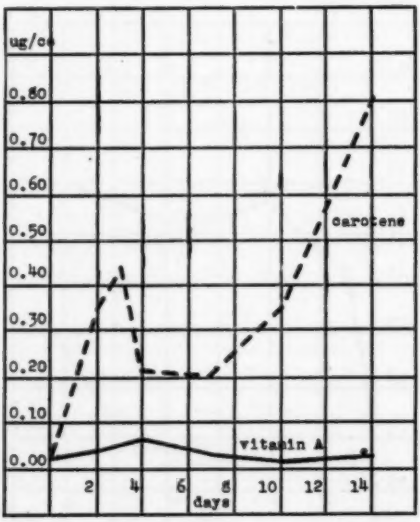

FIG. 2. The effect of carotene on the vitamin A levels of the blood of the young calf.

of vitamin C could be resorted to for the first 10–12 days only. Thereafter it was found necessary to inject the vitamin for it to be effectively recovered in the blood. It is apparent that oral administration after the tenth day resulted in no higher blood plasma concentrations than that of calves which received vitamin C for only the first seven to ten days (fig. 3). The blood plasma levels of vitamin C in these calves present several distinct differences from those of normal calves. It was possible by feeding vitamin C to maintain nearly a 50 per cent higher level in the blood stream for the first two weeks of life.

This however, did not prevent the decrease in blood vitamin C which fell below normal during the third week. During this same interval the vitamin

C of the normal calf rose as shown by figure 3. After this time the calves on the skimmilk diet continued to show lower blood plasma concentrations of vitamin C throughout the six weeks experimental period.

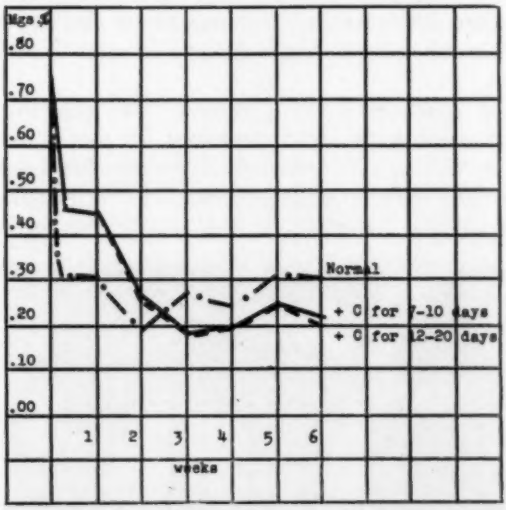

FIG. 3. Relation of oral administration of ascorbic acid to normal levels in the very young calf.

During the course of these experiments several calves with enlarged navels from the regular university herd were placed at our disposal. Blood analyses of these calves indicated them to be low in vitamin C. Daily injections of 0.5 gram ascorbic acid were given and in all cases the navels were completely normal at the end of seven days. The regression in the size of the navel was most rapid in the first 24 to 48 hours. Table 3 shows the

TABLE 3

The effect of ascorbic acid on enlarged navel and peritonitis in the newborn calf

Diet used	Average blood plasma ascorbic acid, mg. per cent	Number of cases of navel inflammation	Ascorbic acid administration	Terminus
Skimmilk only.....	0.6	3	None	2 fatal
Whole milk	0.8	3	½ gram daily for 1 week	Recovered

results of this treatment as well as data on calves receiving the skimmilk diet without added vitamin C. Practical application of this finding has been made on six additional calves with enlarged navels. They were com-

pletely restored to normal by vitamin C injections. These data lend further support to the observation that "navel ill" is controlled where adequate vitamin C is present in the blood of the calf. Navel infections and peritonitis were completely controlled when the blood ascorbic acid values were adequately maintained. Ascorbic acid was highly effective in aiding the body defenses in the control of pneumonia and other types of infectious diseases as well.

The effect of additions of the B-complex. The skimmilk diet supplemented with vitamins A, D, and C supported life, but it did not prevent scours in the very young calf. Since the B-complex had been shown to be necessary in the prevention of scours and that nicotinic, or pantothenic acids or both were specifically suggested (5) experiments were made to determine

FIG. 4. The effects of additions of pantothenic or nicotinic acid to the skimmilk diet of the young calf. The two calves to the left received pantothenic acid while those on the right received nicotinic.

which of these substances would aid in the prevention of scours and if the scours were brought under control would it then be possible to raise the newly dropped calf on vitamin supplemented skimmilk.

The results of these experiments indicated that when the vitamin A levels were adequate and scouring was evident, nicotinic acid (50–100 mg. daily) was effective in helping to control scours (fig. 4). Pantothenic acid fed at the rate of 25–50 mgs. daily had no favorable effect on calf scours. It is also seen that the administration of shark liver oil for vitamin A and mixtures which included nicotinic acid were effective in controlling calf scours (table 4). Practical tests in farm herds have amply verified the correctness of this observation. However, we did experience one condition in which the newborn calf came down with a septicemia and died in 24 to 48 hours. This condition was accompanied by a diarrhea that could not be controlled

by the use of nicotinic acid and vitamin A. With the above data at hand it was found possible to raise Holstein calves on skimmilk supplemented with vitamins A, D, C, and nicotinic acid from the moment they were dropped. We were unsuccessful in raising Guernsey calves under similar treatment. Guernsey, Jersey and Brown Swiss calves were raised on this diet, if they were first allowed to nurse their dams for two or three days.

TABLE 4

Effect of vitamin administration on calf scours

Condition of scours	A, μg./cc. Initial blood plasma-vitamin	Number of cases	Treatment	A, μg./cc. Final blood plasma-vitamin	Remarks
+	0.10	1	vitamin A—(shark liver oil)	0.20	Recovered
+	0.24	1	B complex*	0.09	Not completely recovered
+	0.07	4	B complex + vitamin A	0.17	Recovered
+	0.16	3	B complex only	0.17	Recovered
+	0.07	3	Pantothenic acid + vitamin A	0.06	Died
+	0.11	3	Nicotinic acid + vitamin A	0.17	Recovered
+	0.09	4	Nicotinic acid + pantothenic acid + vitamin A	0.17	Recovered

* B_1, 0.22 gram; riboflavin, 0.22 gram; pantothenic acid, 1.12 grams; choline, 1.12 grams; nicotinic acid, 2.20 grams; B_6, 0.22 gram; and paraaminobenzoic acid, 0.22 gram. This mixture was fed at the rate of 250 mg. per calf per day.

DISCUSSION

The important role of ascorbic acid in the physiology of the calf is shown by these experiments. It was found that low blood plasma ascorbic acid (0.15 mg. per cent or less) accompanied any active infection, and in many cases resulted in navel inflammation and peritonitis. The severity of the infection was greatly relieved if not completely controlled by supplying adequate vitamin C.

The small but definite rise in blood plasma ascorbic acid between the second and third week of life may indicate the beginning of the synthesis of this vitamin. It is interesting to note that the blood plasma ascorbic acid in calves on the skimmilk diet paralleled, but remained lower than that of calves on a normal ration after the second week. This may suggest that there may be a relationship between butter fat in the diet and the ability to synthesize vitamin C. Oral administration is ineffective after about the tenth day of life. This may indicate the beginning of rumen activity, since

it has been shown in older ruminants by Knight *et al.* (2) and others that the vitamin is destroyed in the rumen.

Preliminary evidence with one Holstein calf indicates that very young calves are not able to utilize carotene as a source of vitamin A. With calves on a normal diet a very decided drop in carotene occurred between the second and third week. These observations could be logically interpreted to suggest that carotene conversion was not effective until that period.

SUMMARY

These studies show that vitamin C, A, and nicotinic acid are essential in the diet of the young calf and that they are exceedingly important for the prevention and control of early calfhood diseases.

Specifically the data show that the blood plasma concentration of vitamin C in the new born calf is about twice its normal value. This vitamin has been shown to be highly effective in protecting the calf from navel ill, peritonitis and other active infections. Apparently vitamin C synthesis in the calf begins at about 2 or 3 weeks.

It is possible to maintain the blood plasma vitamin C by oral administration of the vitamin for the first 10 days of life, thereafter it must be injected to be effectively recovered in the blood stream.

On the other hand, the blood concentration of vitamin A and carotene are sufficiently low in the new born calf as to cause it to be vitamin A deficient at birth. It appears unlikely that the very young calf can convert carotene into vitamin A.

It has been demonstrated that adequate vitamin A and nicotinic acid are the essential factors in the control of calf scours; the administration of these substances has controlled all types of scours thus far encountered, except that which accompanied septicemia in the newborn. Pantothenic acid with vitamin A was ineffective in the control of scours in contrast to nicotinic acid.

On the basis of these data Holstein calves can be raised from birth on a diet of skimmilk with added amounts of vitamins A, C, D, nicotinic acid and access to hay and grain.

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OBSERVATIONS ON THE ASCORBIC ACID CONTENT OF EVAPORATED MILK*

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The ascorbic acid content of evaporated milk has been investigated by Meulemans and de Haas (9), who concluded that the canned product contains as much vitamin C as does "prepared" fresh milk. These workers apparently used a direct titration procedure with 2,6-dichlorobenzenoneindophenol and obtained data ranging from 20 to 30 mg. per liter of ascorbic acid in evaporated milk reconstituted to a fluid basis.

Henry, Houston, Kon and Osborne (3), using the precipitation technique of Kon and Watson (8), titrated milk serum with the indophenol dye and found losses of about 30 per cent in the total ascorbic acid content of evaporated milk due to manufacture, with a subsequent gradual loss in storage. Appreciable quantities of dehydroascorbic acid were noted upon reduction of the milk serum with hydrogen sulfide, scrubbing with nitrogen and subsequent titration.

Woessner, Elvehjem and Schuette (13) found only insignificant amounts of dehydroascorbic acid in evaporated milk using a photoelectric method and extrapolating galvanometer readings to zero time, which procedure they found corrected errors due to spurious reducing substances resulting from the effect of hydrogen sulfide on milk ingredients other than dehydroascorbic acid. These workers state that the metallic nature of the manufacturing equipment is not related to the ascorbic acid content of evaporated milk nor are the use of stabilizing salts, the degree of preheating or geographical considerations.

Kon and Henry (7) noted a loss of 43 per cent in the ascorbic acid content of fresh fluid milk as a result of sterilizing at a temperature of 230° F., whereas Josephson and Doan (5) showed that moderately high temperatures (170° F. to boiling) cause a stabilization of the ascorbic acid of milk as a result of heat produced, sulfhydryl, reducing substances derived from certain milk proteins.

PLAN OF STUDY

The study reported here was undertaken to obtain further information relative to the ascorbic acid content of evaporated milk, particularly with respect to factors in manufacturing which may influence its destruction and subsequent stability. This study was made possible by the cooperation of

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the Evaporated Milk Association which furnished samples and complete manufacturing information from 27 plants located in all sections of the country. Four series of samples were supplied in triplicate, in April 1940, December 1940, June 1941, and October 1941, hereafter referred to as series I, II, III, and IV, respectively. One sample in each set was examined when received, another after three months storage at room temperatures, and the third after five or six months from the date of manufacture. Examinations consisted of determinations of total reducing substances (calculated as ascorbic acid), reduced ascorbic acid, dehydroascorbic acid, pH and copper; and a visual appraisal of the degree of spangling of the tin plate of the cans.

Preliminary observations showed that while fluid milk heated to boiling temperature gives a positive nitroprusside test for sulfhydryl compounds, these do not interfere with the direct titration for ascorbic acid by the method of Sharp (11). However, when milk is heated at temperatures and for times comparable to the sterilizing process for evaporated milk (240° F. for 15 minutes) reducing substances reacting with the indophenol dye appear, though the nitroprusside reaction is negative. King (6) states that some sulfhydryl compounds and carbohydrate decomposition products are especially likely to react with the dye and both of these classes of substances are probable in sterilized milk. Direct titration values as high as 60 to 70 mg. per liter calculated as ascorbic acid were noted in freshly sterilized reconstituted evaporated milk. Studies of spontaneous oxidation of evaporated milk and oxidation catalyzed by sunlight and copper indicated that neither the reducing substances other than ascorbic acid, nor ascorbic acid itself, are preferentially oxidized but that both disappear simultaneously following similar smooth curves.

A direct titration procedure for evaporated milk not being possible, a technique involving precipitation of the proteins was studied. A coagulating solution consisting of trichloroacetic acid and metaphosphoric acid suggested by Musulin and King (10) was found suitable but the concentrations were increased to 6 per cent and 4 per cent, respectively, since it appears that all interfering substances are thus filtered out with the proteins and the stability of the ascorbic acid is also improved.

A considerable amount of time was spent in attempting to perfect a satisfactory procedure for determining dehydroascorbic acid in evaporated milk by hydrogen sulfide reduction. This was not wholly successful since it was found impossible to eliminate the difficulty with spurious reducing substances formed by the action of the sulfide on certain milk constituents. Most of these substances are eliminated by precipitation with the coagulating reagent but sufficient remain in the clear serum to cause an almost constant error, equivalent to about one mg. per liter of ascorbic acid when results are compared with those obtained using Esselen and Fuller's (1), "Crook's strain of *E. coli*," as applied to milk by Gunsalus and Hand (2).

METHODS

The cans of evaporated milk were well shaken, opened and immediately diluted with an equal volume of recently boiled distilled water. Total reducing substances were determined at once by direct titration with 2,6-dichlorobenzenoneindophenol dye utilizing a microburette according to the method of Sharp (11). Blanks were obtained by treating a portion of the sample with 5 ppm. of copper (as sulfate) and titrating to constant values. This required from 5 to 8 days. Direct sunlight blanked the samples in much shorter time but was not dependable. Reduced ascorbic acid was determined by treating 25 ml. of the reconstituted milk with 26.7 ml. of coagulant consisting of 6 per cent tricholoracetic acid and 4 per cent metaphosphoric acid (the additional 1.7 ml. being used in order to compensate for the volume of the curd). The coagulum was immediately filtered and 20 ml. of the serum (equivalent to 10 ml. of the reconstituted milk) titrated with the indophenol dye. Blanks for the serum were obtained by treating a portion with 5 ppm. of copper and titrating to constant values. The time required to blank the serum was less than 3 days. Dehydroascorbic acid was determined by treating about 200 ml. of the reconstituted milk with hydrogen sulfide gas for one or two minutes in Erlenmeyer flasks, stoppering, holding for 24 hours, followed by the treatment of 50 ml. with 53.4 ml. of the coagulant, filtering and scrubbing the serum in large test tubes with wet, oxygen-free, nitrogen gas for one hour. Twenty ml. were then titrated with the dye and blanks obtained as mentioned for reduced ascorbic acid. The difference in reducing power as a result of the sulfide treatment was ascribed to dehydroascorbic acid. As mentioned previously, this value for dehydroascorbic acid was found to be in error to the extent of approximately 1 mg. per liter calculated as ascorbic acid. The sulfide method was used because it was found to be considerably less tedious than other methods and because the amounts of dehydroascorbic acid found in samples of evaporated milk were almost negligible. pH values were obtained for the reconstituted samples using a Coleman pH meter and copper analyses were made by the carbamate method of Williams (12) adapted to a photoelectric technique. The tins from which the samples came were carefully washed, dried and held until the end of the storage period, after which they were examined on a comparative basis for etching and spangling of the tin plate.

A study of the loss of ascorbic acid during manufacture and storage was also made by preparing evaporated milk in the laboratory from normal milk and from milk reinforced with ascorbic acid. Examinations were made at various intervals up to nine months.

RESULTS

Data concerning total reducing substances (calculated as ascorbic acid), reduced ascorbic acid and copper for the four series of evaporated milk

samples are presented in summarized form in table 1. These are shown in the form of mean values, ranges, medians, and modes for each series, for each examination period and for milk manufactured in southern plants as compared with that made in northern plants. The date of manufacture and the approximate interval between analyses are also indicated for each series of samples.

Total Reducing Substances

When evaporated milk was reconstituted to a fluid basis and titrated directly with 2,6-dichlorobenzenoneindophenol dye according to the method of Sharp (11), reducing values higher than would be found with fresh fluid milk were generally obtained, agreeing with the findings of Meulemans and de Haas (9) who erroneously concluded that the reducing action under such conditions is entirely attributable to reduced ascorbic acid. More than 85 per cent of the reducing ability was found to be due to substances other than reduced ascorbic acid. These substances could be totally removed by precipitation with a solution of 6 per cent trichloroacetic acid and 4 per cent metaphosphoric acid followed by filtering. The total reducing power of evaporated milk decreased between the third week and the third month of storage and increased between the third month and the fifth or sixth month for no known reason. This was noted, however, in all four series and in most of the individual samples. Some samples gave higher values at five or six months than at three weeks, even though they might have dropped considerably at the three month examination.

Reduced Ascorbic Acid

The greatest amount of reduced ascorbic acid found in any sample analyzed was 10 mg. per liter of reconstituted evaporated milk. The average for all samples, however, was 4.1 mg. per liter which represents a loss of somewhat more than 75 per cent from the average amount found in mixed freshly drawn fluid milk. Compared with average pasteurized bottled milk, as it reaches the consumer, the ascorbic acid content of evaporated milk, on the average, was found to be about one-half as great. It appears from the data shown in table 1 that the greatest loss of ascorbic acid in evaporated milk occurs during manufacture (possibly at the sterilizers) and during the first month or so of storage. Sometime between three weeks and three months after manufacture the ascorbic acid seems to reach a state of stability, little loss occurring thereafter. This stable point may coincide with the exhaustion of oxygen in the can, as may also the rise in total reducing ability of the milk, previously mentioned.

Two other conclusions may be drawn from the data summarized in table 1, neither of which can be readily explained. Samples of evaporated milk in series I and III, manufactured in April and June, respectively, were

TABLE 1

Total reducing substances, ascorbic acid and copper in four series of evaporated milk samples furnished by 27 plants in various parts of the United States. (Reconstituted basis)

Series	Age when examined	Total reducing substances as mg./l. ascorbic acid				Reduced ascorbic acid mg./l.				Copper ppm.			
		Mean	Range	Median	Mode	Mean	Range	Median	Mode	Mean	Range	Median	Mode
I	3 weeks	22.5	14.9–29.1	29.1	21.4	....	.....	....	....	.....	.....	.....	.....
April	3 months	21.0	14.3–26.2	20.3	18.4	5.1	0.9–10.0	4.9	4.9	1.01	0.60–2.60	0.96	1.05
2–5	6 months	26.4	20.4–34.3	26.2	25.2	4.2	0.8– 8.1	3.9	3.9	.....	.....	.....	.....
1940	Series average	23.4	.....	.....	.....	4.6	.....	....	....	.....	.....	.....	.....
II	2 weeks	33.3	22.0–44.5	32.6	28.9	4.6	1.4– 9.5	4.2	3.1	.....	.....	.....	.....
December	3 months	24.9	16.9–33.3	25.1	25.1	3.7	1.1– 7.1	3.0	3.0	0.80	0.38–1.39	0.78	0.81
16–21	5 months	26.4	20.6–33.6	26.0	27.8	3.6	1.2– 6.8	3.2	4.4	.....	.....	.....	.....
1940	Series average	28.5	.....	.....	.....	4.0	.....	....	....	.....	.....	.....	.....
III	3 weeks	26.6	19.0–33.7	27.0	27.7	5.2	1.9– 8.9	5.5	7.0	.....	.....	.....	.....
June	3 months	24.9	18.0–32.0	25.4	25.4	4.2	1.2– 7.9	4.6	6.3	0.70	0.36–1.37	0.59	0.47
10–15	5 months	26.8	20.5–33.1	26.5	26.0	4.2	1.2– 7.3	4.3	6.7	.....	.....	.....	.....
1941	Series average	26.1	.....	.....	.....	4.5	.....	....	....	.....	.....	.....	.....
IV	4 weeks	28.7	21.8–37.8	28.7	27.0	4.3	1.5– 7.4	4.4	6.3	.....	.....	.....	.....
October	3 months	25.4	17.3–32.2	24.5	24.5	3.2	0.8– 6.0	3.2	5.8	0.90	0.37–2.20	0.90	0.75
20–25	6 months	27.1	17.8–34.5	27.0	25.6	3.2	1.0– 6.2	3.1	5.0	.....	.....	.....	.....
1941	Series average	26.8	.....	.....	.....	3.6	.....	....	....	.....	.....	.....	.....
All samples from southern plants						3.5	1.1– 7.1	3.1	1.7	0.82	0.37–1.93	0.75	0.75
All samples from northern plants						4.6	0.8–10.0	4.6	4.4	0.90	0.36–2.60	0.85	0.81

definitely higher in reduced ascorbic acid than those in series II and IV, manufactured in December and October respectively. At the bottom of the table a summary of data obtained from samples manufactured in southern states is compared with that obtained from samples manufactured in northern states. The conclusion seems inescapable that evaporated milk from northern plants contains on the average somewhat greater quantities of ascorbic acid. Whether this is related to geography or dairy practices is a question that cannot be answered from the facts available. This conclusion is not in accord with the findings of Woessner, Elvehjem and Schuette (13).

It was impossible to find any relationship between the reduced ascorbic acid content of evaporated milk and factors encountered in the handling and processing of the product such as: metallic nature of the equipment, forewarming treatment, amount or kind of stabilizing salts used, irradiation or lack of it and sterilizing procedure. In this respect the results confirmed

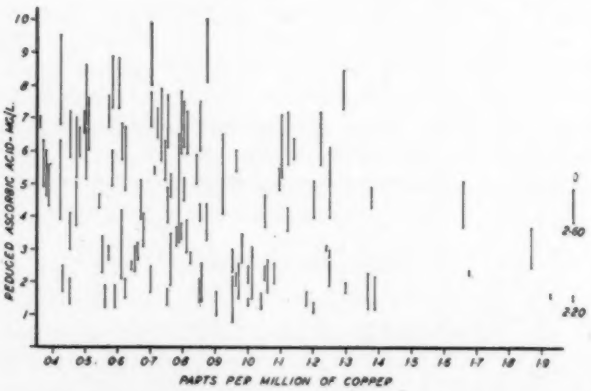

FIG. 1. Lack of relationship between copper content of evaporated milk and amount and stability of reduced ascorbic acid.

those of Woessner, Elvehjem and Schuette (13). Evaporated milk from plants having stainless steel or nickel-chromium equipment throughout exhibited neither greater quantities of reduced ascorbic acid nor increased stability of the vitamin. Nevertheless, it was thought that some relationship might be noted between the copper content of the milk itself and the ascorbic acid.

Copper

In the entire study, the lowest copper content noted was 0.36 ppm., on a reconstituted milk basis, while the highest found was 2.60 ppm. A summary of the copper contents of the samples is shown in table 1. To illustrate more specifically, the lack of relationship between copper content and the amount

and stability of reduced ascorbic acid these values, for each sample in each series, were plotted against each other in figure 1. Each sample is represented by a line located on an ordinate indicating its copper content. The top of the line is on an abscissa representative of its reduced ascorbic acid content at the first examination while the lower end of the line is indicative of the ascorbic acid content at the last examination (5–6 months). Thus, the lines show not only the amount of reduced ascorbic acid, but its relative stability at the various copper levels, the longer lines representing greater loss on holding and the shorter lines lesser loss. The irregular pattern of the lines both as to placement and length is evidence that control of the amount of dissolved copper in evaporated milk (within the ranges observed) is not a factor of outstanding importance in preventing losses of ascorbic acid.

Dehydroascorbic Acid

Evaporated milk samples of series III and IV were analyzed for dehydroascorbic acid by the sulfide method previously described and series IV by the bacteriological technique of Gunsalus and Hand (2). The former method gave results approximately 1 mg. per liter higher than the latter and were inclined to be occasionally erratic and of dubious accuracy. A comparative summary of the results obtained on samples of series IV at the third examination is presented in table 2. If the results obtained by

TABLE 2

Reduced ascorbic acid and dehydroascoric acid content of evaporated milk after six months' storage. (Reconstituted basis)

Series IV six months' examination	Reduced ascorbic acid mg./l.		Dehydroascorbic acid Sulfide method— mg./l.		Dehydroascorbic acid Bacterial method— mg./l.	
	Mean	Range	Mean	Range	Mean	Range
27 samples	3.2	1.0–6.2	1.8	0.1–2.7	0.8	0.0–1.8

the bacterial reduction method are accepted as correct (and these are in conformity with the findings of Woessner, Elvehjem and Schuette using a different method), then it must be concluded that the amount of dehydroascorbic acid in evaporated milk is nutritionally negligible, notwithstanding the fact that in some cases where the amount of reduced ascorbic acid is very low, it may account for upwards of 50 per cent of the total ascorbic acid of the milk.

pH

No relationship was evident between the pH of samples of evaporated milk and their ascorbic acid content, nor between pH and the stability of ascorbic acid. It was noted, however, that the pH of all the samples de-

creased on storage in a rather uniform manner. Table 3 shows in summary fashion a comparison of the drop in pH between milk manufactured in the spring and that manufactured in the autumn. Evaporated milk manufactured in the spring appears to have a lower pH when freshly made but the value does not drop as rapidly as in the case of autumn produced milk which has a higher initial pH.

TABLE 3

Decrease in pH during storage of evaporated milk manufactured in the spring and in the autumn

Season manufactured	Series	Storage period			Decrease
		2 to 4 weeks	3 months	5 to 6 months	
		pH	*pH*	*pH*	*pH*
Spring	I	6.26	6.18	6.15	0.11
	III	6.24	6.20	6.17	0.07
Autumn	II	6.29	6.22	6.16	0.13
	IV	6.28	6.22	6.12	0.16

Spangling of the Tins

Of all the observations made on the samples of evaporated milk in this study, only two exhibited any relationship to each other. The degree of spangling noted on the inside of the tin cans after removing the milk correlated inversely with the copper content of the milk. All cans were examined and some relationship was evident even at the first examination, but it was much more recognizable at the last examination. Judgments of the degree of spangling were considered more accurately made in series III and IV than in I and II, although all four series exhibited similar relationships. Accordingly, only the results obtained with series III and IV samples are shown in table 4 which summarizes the relationship between copper content and spangling. The degree of spangling is indicated in an arbitrary way by the number of plus marks, while the average copper content of the evaporated milk is shown in parts per million. It seems probable that sulfide and sulfhydryl substances, arising through the action of the heat of sterilization on evaporated milk proteins, react with the tin plate to form tin sulfide causing a spangling or etching of the surface (4). If appreciable quantities of copper are present in the milk, the sulfides and sulfhydryls may be partially oxidized and rendered less active in attacking the tin of the cans.

STUDIES WITH LABORATORY PREPARED EVAPORATED MILK

A small batch of fluid whole milk containing 13.9 mg. per liter of reduced ascorbic acid was forewarmed at 202° F. for 10 minutes without affecting the ascorbic acid content. It was concentrated to a solids-not-fat content of approximately 18 per cent and homogenized. The ascorbic acid dropped

to 12.2 mg. per liter. The batch was divided into two parts, one of which was reinforced with pure ascorbic acid to the extent of 25 mg. per liter (fluid basis). Both portions were filled into tins and sterilized at 240° F. for 15

TABLE 4

Inverse relationship between copper content of evaporated milk and degree of spangling of the cans after 5–6 months' storage
(Series III and IV)

Degree of spangling	±	+	++	+++	++++
	Copper Content ppm.				
Series III	–	.97	.89	.54	.51
Series IV	2.20	1.01	.96	.70	.49

minutes. As a result of sterilization, the ascorbic acid fell from 12.2 to 8.3 mg. per liter in the normal milk and from 36.4 to 26.1 mg. per liter in the reinforced milk. Samples of the two lots of milk were examined at intervals up to nine months of storage at room temperature. In the first samples

TABLE 5

Loss of reduced ascorbic acid in evaporated milk and reinforced evaporated milk due to concentration, sterilization and storage for nine months.
(Reconstituted basis)

Time examined	Normal sample			Sample reinforced 25 mg./l. with ascorbic acid		
	Reduced ascorbic acid		Dehydro-ascorbic acid (bacterial)	Reduced ascorbic acid		Dehydro-ascorbic acid (bacterial)
	mg./l.	*% loss*	*mg./l.*	*mg./l.*	*% loss*	*mg./l.*
Raw milk	13.9	.....	3.0	.....	.....	.....
Forewarmed (dilution)	12.7	.....	0.9	.....	.....	.....
Forewarmed (corrected)	14.0	.....	1.0	.....	.....	.....
Concentrated and homogenized	12.2	0.0	1.8	36.4	0.0	1.8
Sterilized	8.3	32.0	0.1	26.1	28.3	0.3
24 hours after sterilization	7.2	40.9	0.1	24.8	31.9	0.1
48 hours storage	7.0	42.3	0.4	24.2	33.5	0.1
5 days storage	6.3	48.3	.....	23.1	36.5	.....
9 days storage	5.7	52.3	.....	21.7	40.8	.....
3 weeks storage	5.0	59.0	.....	19.6	46.3	.....
5 weeks storage	4.0	67.2	.....	17.8	51.1	.....
7 weeks storage	4.0	67.2	.....	16.7	54.3	.....
3 months storage	4.0	67.2	.....	16.8	53.9	.....
7 months storage	4.1	66.4	.....	16.5	54.7	.....
9 months storage	4.2	65.6	.....	15.9	56.3	.....

examined determinations of dehydroascorbic acid were made, by the bacterial method (2), but these were discontinued since insignificant amounts were found in the heated milk. Table 5 exhibits the data obtained in this study.

In both the normal and the reinforced evaporated milk the ascorbic acid content dropped slowly but continuously until between the fifth and seventh weeks of storage, at which time the normal milk had lost 67.2 per cent of the amount present when sterilized and the reinforced milk had lost between 51.1 and 54.3 per cent. No further significant losses in ascorbic acid occurred, indicating that an equilibrium is reached which may correspond with the exhaustion of oxygen in the can. Apparently no quantitative relationship exists between the amount of oxygen in the can and the amount of reduced ascorbic acid oxidized, inasmuch as the cans were all filled to about the same degree, yet more than twice as much ascorbic acid was oxidized in the case of the reinforced evaporated milk than in the case of the normal evaporated milk, notwithstanding the fact that the percentage loss was less. If means could be developed to reduce the loss of ascorbic acid occurring before the equilibrium point is reached, it might be feasible and desirable to reinforce all evaporated milk with ascorbic acid. Studies dealing with this possibility are now being conducted.

CONCLUSIONS

The direct titration method of Sharp (11) for determining reduced ascorbic acid is not applicable to evaporated milk because of the spurious reducing substances formed during sterilization, but a precipitation procedure employing trichloroacetic acid and metaphosphoric acid removes these substances along with the protein and titration of the serum gives satisfactory results.

No methods were found satisfactory for overcoming the errors inherent in the hydrogen sulfide reduction procedure for estimating the amount of dehydroascorbic acid in evaporated milk when these were compared with results obtained using the bacterial procedure of Gunsalus and Hand (2).

The amount of dehydroascorbic acid in evaporated milk at any time after sterilization is nutritionally insignificant and this determination can be omitted without appreciable error.

There is no relation between the metallic nature of the equipment used for processing evaporated milk, the forewarming treatment, the amount and kind of stabilizing salts employed, irradiation or the sterilizing process and the amount or stability of ascorbic acid contained in the product.

The amount and stability of ascorbic acid in evaporated milk is not related to the copper content, contrary to expectations.

Evaporated milk manufactured in the spring and early summer appears to contain more ascorbic acid than that made in the fall and early winter, as does also that produced in northern plants as compared with similar milk manufactured in plants located in southern sections of the country.

The greatest loss of ascorbic acid apparently takes place before, during and immediately after the manufacture of evaporated milk, a further

gradual loss occurring during the first two months of storage. After approximately six weeks to two months of storage, however, little further loss occurs up to nine months of storage and presumably longer.

Spangling or etching of the tin of evaporated milk cans is invariably observable after two months of storage. The degree of spangling is inversely correlated with the copper content of the milk.

The pH of evaporated milk decreases during storage, but milk manufactured in the spring and summer appears to be slightly better buffered than that made in the fall and winter.

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RENNIN ACTION IN RELATION TO THE WATER BINDING AND ELECTROKINETIC PROPERTIES OF CALCIUM AND SODIUM CASEINATE SOLS*

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Söhngen, Wieringa and Pasveer (9) proposed a theory supported by viscometry evidence that a certain amount of dehydration of the casein explains the action of rennin as far as the initial stage of the rennin coagulation process is concerned. Richardson and Palmer (7) have shown that rennin decreases the electrokinetic charge on the casein micelles, and since Smoluchowski (8) has shown that a suspension of charged particles is more viscous than one of uncharged particles, the reality of a true dehydration effect is open to question.

Briggs (1) and Hankinson and Briggs (3) investigated the hydration of sodium gum arabic and sodium and calcium caseinate systems by applying the Krasny-Ergen (5) equation for electroviscous effect, which interrelates hydration and the electrokinetic potential. The viscosity relationships in the equation were verified by data on these colloidal systems, but a colloid concentration factor appears to be necessary for proper evaluation of the radius of the micelles. The order of magnitude and fundamental reason for the necessity of this factor have not been established, so the casein concentration was held constant in the experiments which follow.

It was the purpose of the investigation reported in this paper to extend the study of the electroviscous effect to rennin-treated caseinate systems with the view of trying to determine whether a true dehydration really occurs or if the decrease in viscosity is accounted for by the decrease in electrokinetic charge. In addition a comparison was made with the effects of pepsin, a highly proteolytic as well as milk clotting enzyme.

The rennin used in this study was the purified product described by Hankinson and Palmer (4) and was freshly dialyzed as needed. It possessed four and one-half times the milk clotting activity of the original rennet extract[1] on a salt-free dry basis and only seven per cent of the original proteolytic activity. The use of this rennin should minimize effects which might be due to proteolysis or to impurities present in the rennet extract. The pepsin used was an Armour 1 : 10,000 U.S.P. product.

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[1] Generous supplies of rennet extract were kindly furnished by the Chr. Hansen's Laboratories, Milwaukee, Wisconsin.

The previously cited references (1) and (3) should be referred to for a discussion of the experimental limitations and applications of the equation for the electroviscous effect. The equation in its linear form is repeated here for ready reference.

$$\frac{\eta_0}{\eta_s - \eta_0} = \frac{1}{\eta_{sp}} = -\frac{3\varepsilon^2}{8\pi^2 r^2} \cdot \frac{\zeta^2}{\lambda(\eta_s - \eta_0)} + \frac{1}{K\phi}$$

Measurements were taken of electrokinetic potential (ζ) specific conductivity (λ) and relative viscosity $\frac{\eta_s}{\eta_0}$ and the data were calculated and plotted with $\frac{1}{\eta_{sp}}$ and $\frac{\zeta^2}{\lambda(\eta_s - \eta_0)}$ as coordinates after converting to electrostatic units. It was possible in this manner to evaluate the volume per cent (ϕ) occupied by the dispersed phase from the Y-intercept, using the Einstein constant 2.5 for K. Theoretically, it should be possible to evaluate the average radius (r) of the particle from the slope of the plot, using the dielectric constant (ε) of water at 30° C.

The experimental conditions and methods used were identical with those described previously for sodium gum arabic and caseinate systems. A temperature of 30° C. was employed for the viscosity and conductivity measurements using a thermostatically controlled water bath. A temperature of 28–30° C. was employed for the migration measurements, this being the range of fluctuation in room temperature. All mobility data were corrected to 30° C. in the usual manner according to the viscosity ratio of water at the two temperatures.

The purified, electrodialyzed, 100-mesh casein was prepared according to the Van Slyke (10) modification of the Van Slyke and Baker (11) method. Sols were prepared by triturating the dry casein with 15 ml. of saturated calcium hydroxide solution per gram of casein or five ml. of N/10 sodium hydroxide solution per gram of casein, bringing the pH to approximately 6.6. The sols were then diluted to the desired concentration (2.5 per cent casein), stirred mechanically for one-half hour and used within a few hours after preparation.

Artificial "milks" were prepared by dissolving the casein in an excess of calcium hydroxide solution, cooling to 10° C., titrating back with one per cent H_3PO_4 solution accompanied by rapid mechanical stirring, aging at 2° C. over night and dialyzing for 24 hours in "Visking" casings at 6° to 7° C., using one change of water (ten volumes) after about six hours. One "milk" sample was removed at a time from the cold, warmed to room temperature, the necessary addition of salt or rennin made and the sample held for one-half hour. All the measurements were then taken before removing the next sol from the cold. Variations due to hysteresis were minimized in this manner.

No attempt was made in this study to determine the effect of varying the hydrogen ion concentration, but the pH was measured for all samples, using a Coleman pH electrometer.

EXPERIMENTAL RESULTS

Effects of $CaCl_2$ and Rennin on Calcium Caseinate Sols

The experimental data showing the effects of adding $CaCl_2$ and rennin to calcium caseinate sols are recorded in table 1. Calculations from the data are plotted in figure 1 for the purpose of evaluating ϕ and r in studying the electroviscous effect.

TABLE 1

The effects of $CaCl_2$ and rennin on calcium caseinate

Experimental variable	pH	λ $\times 10^{-4}$ mhos.	μ/sec. / volt/cm. cm. $\times 10^{-4}$*	ζ $-$ mv.	$\frac{\eta_s}{\eta_0}$	$\frac{\zeta^2}{\lambda(\eta_s - \eta_0)}$ E.S.U. $\times 10^{-14}$ †	$\frac{1}{\eta_{sp}}$
I. Calcium caseinate and varying amounts of $CaCl_2$(N) ‡							
0.0 N	6.63	2.39	1.54	17.4	1.605	0.323	1.65
0.0005 N	6.63	2.81	1.50	17.0	1.555	0.286	1.80
0.001 N	6.63	3.26	1.52	17.2	1.478	0.293	2.09
0.002 N	6.63	4.24	1.40	15.8	1.358	0.254	2.79
0.005◊ N	6.50	7.35	1.23	13.9	1.253	0.160	3.95
II. Calcium caseinate and varying amounts of rennin (R) ‖							
10^{-4} R	6.63	2.41	1.52	17.2	1.429	0.442	2.33
10^{-3} R	6.63	2.41	1.45	16.4	1.324	0.532	3.09
10^{-2} R	6.63	2.45	1.45	16.4	1.305	0.556	3.28
10^{-1} R	6.63	2.69	1.34	15.2	1.280	0.473	3.57
Undil. R	6.51	3.18	1.23	13.9	1.259	0.362	3.86
III. Calcium caseinate and undiluted rennin at varying reaction time (min.)							
0.0 min.	6.63	2.39	1.55	17.5	1.620	0.319	1.61
2.5 min.	.....	2.52	1.40	15.8	1.357	0.429	2.80
5.5 min.	.....	2.56	1.37	15.5	1.334	0.434	3.00
8.0 min.	.....	2.60	1.34	15.2	1.327	0.420	3.06
11.0 min.	.....	2.65	1.33	15.0	1.320	0.410	3.13
20.0 min.	.....	2.76	1.31	14.8	1.315	0.389	3.18
30.0 min.	.....	2.85	1.30	14.7	1.310	0.378	3.23

* Migration rates measured at room temperature 28° C. and corrected to 30° C.
† Conversion factor for E.S.U. = 1.235×10^{-2}.
‡ N is the normality concentration of $CaCl_2$ in the final solution.
◊ Higher concentrations of $CaCl_2$ caused a precipitate.
‖ 1.0 ml. solution at the concentration stated added per 100 ml. sol. and allowed to stand for 2½ hrs. before taking measurements.

Figure 1 shows that $CaCl_2$ and rennin additions to calcium caseinate sols have entirely different effects on the relations between the relative viscosity function expressed as $\frac{\eta_0}{\eta_s - \eta_0}$ and the ζ-potential function expressed as

$\frac{\zeta^2}{\lambda(\eta_s - \eta_o)}$. A linear relationship with a negative slope (graph BA) as demanded by the Krasny-Ergen equation is fulfilled when $CaCl_2$ is added to calcium caseinate sols as shown in a previous paper (3). Upon extrapolation to the Y axis the value for ϕ is found to be 6.45 when $K = 2.5$ and the concentration of dry casein is 2.5 grams per 100 ml. Considerable water has evidently been taken up into the micelle, causing swelling. The electroviscous effect is shown to be surprisingly high, the relative viscosity of the sol being 1.605 as compared with a calculated value of 1.161 in the absence of electroviscous effect.

In the study of the effect of different concentrations of rennin the lower amounts of rennin result in the relative viscosity–ζ-potential relation having a positive instead of a negative slope (graph AC) required for a true electroviscous effect by the Krasny-Ergen equation. Even the lowest concentration of rennin employed causes a rapid increase in the viscosity function with a slight increase rather than a decrease in the ζ-potential function. A point is reached, however, where the slope of the graph reverses and a true electroviscous relationship again develops (graph CD). However, the slope of the new electroviscous relation is more horizontal than found in the case of adding $CaCl_2$ to the caseinate sol, thus indicating a larger particle size. The $\frac{1}{K\phi}$ value obtained by extrapolation to the Y axis is lower than in the case of adding $CaCl_2$ to the sol, indicating a larger value for ϕ (upon calculation $\phi = 8.00$). Here are two indications that a larger particle exists after rennin has acted than existed originally even though a marked decrease in relative viscosity has occurred.

Calculations for the radius and number of particles have been omitted as they are only relative and cannot be taken as absolute values because: 1) a colloid concentration factor is missing from the electroviscosity equation; 2) considerable variation has been found between sols even when the greatest effort is made to keep all factors constant; 3) errors in measurements may become magnified when so many variables are considered in the equation; and 4) many assumptions are made in deriving the electroviscosity equation which may or may not be fulfilled by these particular systems studied.

The data in part III, table 1, in which one ml. of undiluted, dialyzed rennin was added to 100 ml. of calcium caseinate sol, and measurements made at stated intervals of time, show the same type of graphic relationship as part II (fig. 1, graph AEF). The difference in the relationship probably lies in the fact that 2.5 hours were allowed for attaining equilibrium in part II, consequently the extent of the reaction was increased. It appears that the same course of action is followed whether a high concentration of rennin acts for a short time or whether lower concentrations of rennin are allowed to react for a longer time and proceed to equilibrium.

It was established that the trace of NaCl remaining in the dialyzed rennin solution did not contribute to the changes evidenced when rennin was added to the caseinate sols.

It was found impossible to show the electroviscous effect of both $CaCl_2$ and rennin on the same sample, even at the lower concentrations, because of the formation of precipitates.

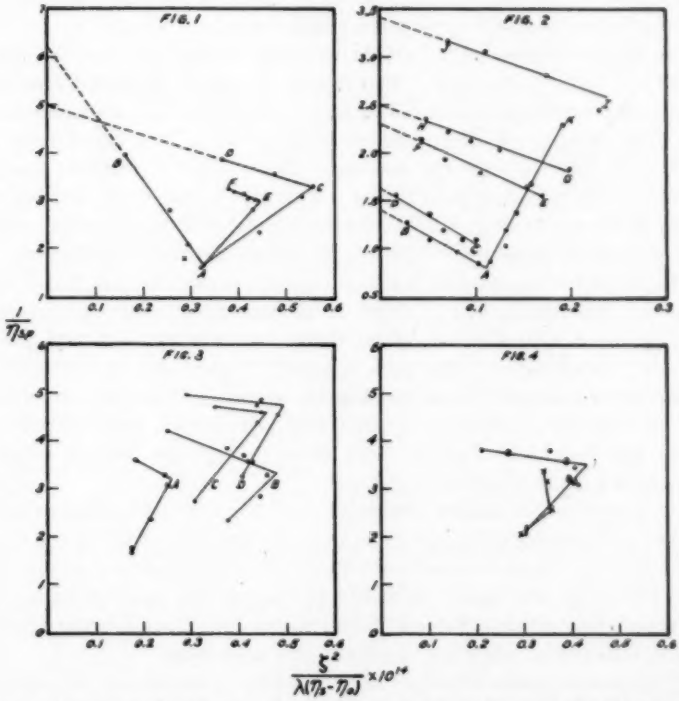

FIG. 1. The effects of $CaCl_2$ and rennin on calcium caseinate. Graph AB = varying amounts of $CaCl_2$; graph ACD = varying amounts of rennin; graph AEF = undiluted rennin at varying reaction time (min.).

FIG. 2. The effects of NaCl and rennin on sodium caseinate. Graph AB = varying amounts of NaCl; graph AK = varying amounts of rennin; graph CD = varying amounts of NaCl alone; graph EF = 10^{-3} rennin and varying amounts of NaCl; graph GH = 10^{-1} rennin and varying amounts of NaCl; graph IJ = undiluted rennin and varying amounts of NaCl.

FIG. 3. The effects of varying amounts of rennin on dialyzed artificial "milks." The letters on the graphs correspond to "milks" with varying composition shown in table 3.

FIG. 4. Comparison of effects of purified rennin, rennet extract and pepsin on calcium caseinate. ● = purified rennin; ⊙ = rennet extract; x = pepsin.

The Effects of NaCl and Rennin on Sodium Caseinate

The measurements and calculations from the data for sodium chloride and rennin added singly to a sodium caseinate sol are presented in table 2 and graph BAK, figure 2. Several striking differences are noted between sodium and calcium caseinate sols at the same pH. The former yields a clear sol while the latter is milky. The relative viscosities are 2.159 and 1.605, ζ-potentials -24.4 and -16.6 millivolts and the specific conductivities 7.57×10^{-4} mhos and 2.39×10^{-4} mhos respectively.

The Krasny-Ergen equation fits the data fairly well for the electroviscosity of sodium caseinate. The relative viscosity decreased from 2.159 to an extrapolated value of 1.725 at zero ζ-potential. The calculated value for ϕ in the absence of electroviscous effect is 29.0. This value when compared with 6.45 for calcium caseinate, shows that the sodium caseinate micelle is much more highly hydrated. This may be due to the greater penetration of the monovalent NaOH into the micelle with the resulting osmotic effect of drawing more water into the micelle to establish equilibrium.

The graph for the effect of rennin on sodium caseinate (AK, fig. 2) shows a positive slope throughout. There is no reversal of slope corresponding to an apparent agglutination of particles as was evidenced with calcium caseinate. Examination of the data in table 2, Experiment II, indicates that the phenomenon appears to be principally one of dehydration accompanied by a decrease in ζ-potential. Both stabilizing factors were reduced materially but they were both so high initially that the critical point for agglutination was not attained.

The charge and hydration of sodium caseinate were still sufficiently large after the most concentrated rennin had acted to permit a study of the electroviscous effect of the addition of NaCl to the sols. The purpose of this study was to determine the change in radius of the particle and the value for ϕ after rennin had acted. Table 2, Experiments III–VI and figure 2, graphs CD, EF, GH and IJ, show the results of this experiment.

Although the points do not all fall on straight lines, it may be noted that the slopes of the lines are becoming more horizontal as the amount of rennin is increased (compare line IJ with line CD). It may be assumed that the rennin dehydrates the particles materially and when NaCl is added to this partially dehydrated system an agglutination of colloidal particles is brought about. This would account for the more horizontal slope and larger radius. The sols in Experiment VI were all very milky in appearance and undoubtedly some agglutination had occurred. Both the relative viscosity at zero ζ-potential and the calculated ϕ show a marked decrease with increasing rennin concentration as contrasted with the results on calcium caseinate sols.

Effect of Rennin on Calcium Caseinate—Calcium Phosphate "Milks"

Four "milks" were prepared according to the method already described. At first considerable difficulty was encountered with instability but it was

found that if the titration and dialysis were carried out at 7–10° C. satisfactory stability resulted. Dialysis was necessary to eliminate the clotting phase and obtain interpretable results.

TABLE 2

The effects of NaCl and rennin on sodium caseinate

Experimental variable	pH	λ ($\times 10^{-4}$ mhos.)	μ/sec. volt/cm. (cm. $\times 10^{-4}$)	ζ (– mv.)	$\frac{\eta_s}{\eta_o}$	$\frac{\zeta^2}{\lambda(\eta_s - \eta_o)}$ (E.S.U. $\times 10^{-14}$)	$\frac{1}{\eta_{sp}}$
I. Sodium caseinate and varying amounts of NaCl (N)							
0.0 N	6.63	7.57	2.26	25.6	2.159	0.115	0.862
0.001 N	6.63	8.71	2.30	26.0	2.180	0.102	0.847
0.005 N	6.63	12.58	2.32	26.2	2.039	0.081	0.962
0.01 N	6.63	18.42	2.08	23.5	1.912	0.051	1.096
0.02* N	6.55	28.89	1.74	19.7	1.790	0.027	1.266
II. Sodium caseinate and varying amounts of rennin (R)							
10^{-4} R	6.63	7.66	2.22	25.1	1.961	0.132	1.041
10^{-3} R	6.63	7.80	2.08	23.5	1.834	0.131	1.200
10^{-2} R	6.61	7.80	2.02	22.8	1.727	0.142	1.376
10^{-1} R	6.58	7.80	1.93	21.8	1.596	0.158	1.678
Undil. R	6.50	7.80	1.82	20.6	1.437	0.192	2.290
III. Sodium caseinate with varying amounts of NaCl (N)							
0.0 N	6.62	7.79	1.89	21.4	1.910	0.100	1.099
0.001 N	6.62	8.93	1.88	21.3	1.921	0.085	1.086
0.005 N	6.57	12.92	1.88	21.3	1.831	0.065	1.203
0.01 N	6.57	18.37	1.85	20.9	1.734	0.050	1.362
0.02 N	6.50	27.57	1.20	13.6	1.652	0.016	1.534
IV. Sodium caseinate and 10^{-3} rennin with varying amounts of NaCl (N)							
0.0 N	6.62	7.82	2.07	23.4	1.642	0.170	1.558
0.001 N	6.63	8.94	2.04	23.1	1.601	0.155	1.664
0.005 N	6.57	13.44	1.98	22.4	1.559	0.104	1.789
0.01 N	6.57	19.23	1.85	20.9	1.518	0.068	1.931
0.02 N	6.48	29.91	1.71	19.4	1.463	0.042	2.160
V. Sodium caseinate and 10^{-1} rennin with varying amounts of NaCl (N)							
0.0 N	6.61	7.94	2.08	23.5	1.548	0.198	1.825
0.001 N	6.59	8.97	1.66	18.8	1.492	0.125	2.033
0.005 N	6.55	13.34	1.73	19.6	1.471	0.095	2.123
0.01 N	6.50	19.00	1.74	19.8	1.451	0.071	2.217
0.02 N	6.46	29.56	1.72	19.5	1.424	0.047	2.358
VI. Sodium caseinate and undiluted rennin with varying amounts of NaCl (N)							
0.0† N	6.55	7.97	1.93	21.9	1.407	0.230	2.457
0.001 N	6.51	9.04	1.68	19.0	1.355	0.175	2.817
0.005 N	6.47	13.49	1.56	17.6	1.327	0.109	3.058
0.02 N	6.43	19.20	1.47	16.7	1.316	0.071	3.165

* Higher concentrations of NaCl caused bubbling at electrode of the cataphoresis cell.

† There was a gradual increase in turbidity from III to VI; all samples of lot VI were very milky.

Each lot of "milk" contained five grams of casein with varied amounts of $Ca(OH)_2$ and H_3PO_4. All were diluted to 200 ml. The volumes of saturated $Ca(OH)_2$ solution present in milks A, B, C and D, table 3, were 75, 100, 125, and 150 ml., respectively. The volumes of one per cent H_3PO_4 solution were 0, 8.5, 14.5, and 19.5 ml. respectively. Figure 3 shows the

TABLE 3

The effects of rennin on dialyzed artificial milks

Conc. rennin added	pH	λ $\times 10^{-4}$ mhos.	$\frac{\mu/\text{sec.}}{\text{volt/cm.}}$ em. $\times 10^{-4}$	ζ $-$ mv.	$\frac{\eta_s}{\eta_0}$	$\frac{\zeta^2}{\lambda(\eta_s - \eta_0)}$ E.S.U. $\times 10^{-14}$	$\frac{1}{\eta_{sp}}$
A. 5 gm. casein and 75 ml. saturated $Ca(OH)_2$, diluted to 200 ml.							
0	6.55	2.84	1.19	13.5	1.576	0.173	1.736
10^{-4}	6.55	2.86	1.22	13.8	1.596	0.173	1.678
10^{-3}	6.55	2.83	1.14	12.9	1.425	0.215	2.353
10^{-2}	6.55	2.95	1.10	12.4	1.327	0.248	3.058
10^{-1}	6.52	3.17	1.09	12.3	1.306	0.242	3.268
Undil.	6.47	3.66	0.96	10.9	1.278	0.182	3.597
B. 5 gm. casein, 100 ml. saturated $Ca(OH)_2$, 8.5 ml. 1% H_3PO_4, diluted to 200 ml.							
0	6.35	2.51	1.43	16.2	1.430	0.377	2.326
10^{-4}	6.35	2.50	1.40	15.9	1.353	0.445	2.833
10^{-3}	6.35	2.51	1.33	15.0	1.305	0.457	3.279
10^{-2}	6.35	2.54	1.22	13.8	1.281	0.414	3.559
10^{-1}	6.31	2.68	1.15	13.0	1.262	0.374	3.817
Undil.	6.25	3.07	0.95	10.8	1.238	0.249	4.202
C. 5 gm. casein, 125 ml. saturated $Ca(OH)_2$, 14.5 ml. 1% H_3PO_4, diluted to 200 ml.							
0	6.50	2.89	1.28	14.4	1.362	0.307	2.762
10^{-4}	6.50	2.92	1.28	14.4	1.270	0.408	3.704
10^{-3}	6.50	2.90	1.21	13.7	1.229	0.439	4.367
10^{-2}	6.48	2.89	1.19	13.5	1.218	0.448	4.587
10^{-1}	6.43	2.96	1.06	11.9	1.213	0.350	4.695
Undil.	6.35	3.30	0.91*	10.3	1.226*	0.221	4.425
D. 5 gm. casein, 150 ml. saturated $Ca(OH)_2$, 19.5 ml. 1% H_3PO_4, diluted to 200 ml.							
0	6.57	2.52	1.25	14.2	1.308	0.405	3.247
10^{-4}	6.55	2.49	1.22	13.8	1.280	0.424	3.571
10^{-3}	6.55	2.47	1.15	13.0	1.221	0.481	4.525
10^{-2}	6.53	2.50	1.08	12.2	1.212	0.437	4.717
10^{-1}	6.51	2.68	1.11	12.6	1.207	0.445	4.831
Undil.	6.48	3.11	0.95	10.8	1.202	0.289	4.950

* Partial clot.

effects of different concentrations of rennin on the relation between the relative viscosity and ζ-potential functions in these "milks."

It is noticed that "milk" B had a considerably lower pH value than the other three samples. Too much phosphoric acid was added in titrating back to a pH below 6.6 and dialysis did not return the pH to the value desired. The effect of the low pH is reflected in the position of the graph being to the right of normal in figure 3.

The data for η_s, λ, ζ and pH of samples A, C and D in table 3 show that in the absence of rennin the η_s decreases with no corresponding regularity of change in λ, ζ and pH as the proportion of calcium phosphate is increased. It is evident that the addition of the hydrophilic colloid to the system does not produce a change in its electroviscosity. It appears probable therefore that the decrease in relative viscosity must be due either to a change in hydration or to a change in the shape of the particle or both. Evidence is not available to determine which of these alternatives is involved.

The series of graphs in figure 3 demonstrates that the mode of action of rennin is the same on artificial "milks" as on simple calcium caseinate systems, even though the initial viscosity is markedly lower in these systems.

A further observation of importance in this study is the progressive decrease in electroviscous slope as the concentration of colloidal calcium phosphate increases. The interpretation is that the radius of the aggregate is larger at the higher calcium phosphate concentrations. It cannot be stated whether this is larger because of the size of the individual particles in the aggregate or because of the number present.

The Effect of Rennin on Skim Milk

It is desirable to know whether the experimental approach to the problem of rennin action, employed in the foregoing experiments, is applicable to the more complex natural systems existing in milk. Several difficulties are presented. An attempt was made to eliminate the major ones by throwing out fat globules and coarsely suspended material in a Sharples Supercentrifuge, removing the free calcium by dialysis to prevent clotting when rennin is added and by making the caseinate system visible through addition of finely powdered quartz, on which the caseinate would be expected to be adsorbed. Various concentrations of rennin were added to samples of skim milk treated in this manner and the ζ-potential, relative viscosity and specific conductivity measured. Although the changes observed were in the direction anticipated, they were not only small but the observed ζ-potentials were surprisingly low. As to the latter Moyer (6) has pointed out certain discrepancies in migration studies when employing protein-coated inert particles. In general, it can be concluded that experimental limitations prevent the use of natural milk for demonstrating the effects of rennin on the hydration and electroviscosity of its caseinate sols.

Comparison of Effects of Purified Rennin, Rennet Extract and Pepsin on Calcium Caseinate

A comparison of the effect of purified rennin, rennet extract and pepsin was made using enzyme solutions having equal concentrations of dry solids. The dialyzed purified rennin and rennet extract solutions were diluted to contain 0.6 per cent dry solids and an 0.6 per cent pepsin solution was prepared from the Armours 1 : 10,000 dry scale preparation. These solutions

then constituted stock solutions and were diluted logarithmically and added in one per cent proportions to a 2.5 per cent calcium caseinate sol.

The results of this study are shown in table 4 and figure 4. The only difference between the effects of highly purified rennin and rennet extract appears to be that of magnitude. Both the ζ-potential and η_s decreased more and the λ increased more when purified rennin was used than when

TABLE 4

Comparison of effects of purified rennin, rennet extract and pepsin on calcium caseinate

Conc. enzyme added	pH	λ $\times 10^{-4}$ mhos.	μ/sec. volt/cm. cm. $\times 10^{-4}$	ζ $-$mv.	$\frac{\eta_s}{\eta_0}$	$\frac{\zeta^2}{\lambda(\eta_s - \eta_0)}$ E.S.U. $\times 10^{-14}$	$\frac{1}{\eta_{sp}}$
			I. Purified rennin				
0	6.72	2.42	1.33	15.0	1.484	0.299	2.066
10^{-4}	6.72	2.47	1.31	14.8	1.454	0.303	2.203
10^{-3}	6.72	2.47	1.29	14.6	1.324	0.413	3.086
10^{-2}	6.72	2.47	1.21	13.7	1.291	0.406	3.436
10^{-1}	6.66	2.56	1.10	12.4	1.264	0.353	3.788
Undil.	6.60	2.87	0.89	10.1	1.263	0.210	3.802
			II. Rennet extract diluted 6 to 10				
10^{-4}	6.73	2.41	1.24	14.1	1.319	0.402	3.135
10^{-3}	6.72	2.47	1.14*	12.9*	1.337	0.310	2.967
10^{-2}	6.72	2.47	1.23	14.0	1.313	0.393	3.195
10^{-1}	6.69	2.52	1.17	13.3	1.281	0.388	3.559
Undil.	6.65	2.65	0.97	11.0	1.268	0.265	3.731
			III. Pepsin 0.6% solution				
10^{-4}	6.70	2.41	1.30	14.7	1.460	0.303	2.174
10^{-3}	6.72	2.45	1.32	14.9	1.482	0.292	2.075
10^{-2}	6.72	2.47	1.32	14.9	1.391	0.357	2.558
10^{-1}	6.70	2.50	1.17	13.3	1.316	0.348	3.165
Undil.	6.68	2.59	1.15	13.0	1.297	0.341	3.367

* Result is anomalous, possibly due to a leak at stopcocks of the cataphoresis cell.

rennet extract was used. However, the same graph fits both sets of data reasonably well. This difference in magnitude of action when measured by ζ-potential, η_s and λ methods is to be expected since the purified rennin was known to possess four and one-half times the milk clotting activity of the rennet extract on a dry solids basis.

The data for the action of pepsin on calcium caseinate indicate that a destabilization of the caseinate micelle is effected but in an entirely different manner and to a lesser extent than by rennin. Both the ζ-potential and η_s decrease but not to the point of aggregation of the micelles. If relatively more pepsin had been used, aggregation would undoubtedly have resulted, because it is known that pure crystalline pepsin possesses milk coagulating properties. The concentration of pepsin required to reach the zone of aggregation appears to be much greater than that for rennin. It is concluded

that the small amount of pepsin present in the rennet extract employed does not influence the results obtained when crude rennet extract is used for a study of the action of rennin on calcium caseinate. Crystalline rennin, isolated by one of us (2) after this work was undertaken, would be expected to differ from the highly purified rennin only in the magnitude of its effect.

The ζ-Potential of Rennin at Different pH Levels

It has recently been shown (2) that the isoelectric zone for crystalline rennin is pH 4.45 to 4.65 and that the colloidal protein particles possess a negative charge on the alkaline side of the isoelectric point. However, it was considered possible that the magnitude of this charge could be considerably less than that on the caseinate; thus the mixture of two colloids of different intensity of electric charge would tend to seek equilibrium at some charge intermediate between the two. It was therefore deemed necessary to determine the magnitude of the ζ-potential of the rennin micelle at different levels of pH.

It was found that the dialyzed rennin could be diluted ten times and still possess enough visible particles under the microscope to follow the cataphoretic mobility. The pH value of the diluted dialyzed rennin was 6.0; therefore N/10 NaOH was added to adjust the pH levels to 6.5, 7.0 and 7.5. The results of this study are shown in table 5.

TABLE 5

The ζ potential of rennin at different pH levels

pH	$\frac{\mu/sec.}{volt/cm.}$	ζ
	cm. × 10^{-4}	− *mv.*
6.0	0.93	10.5
6.5	1.32	14.9
7.0	1.76	19.9
7.5	1.95	22.1

The − 14.9 mv. ζ-potential of rennin at pH 6.5 corresponds very closely to that of a calcium caseinate sol at this pH. The latter sols in the studies already described, showed a ζ-potential range of − 13 to − 16 mv. between pH 6.5 and 6.7. There is a progressive increase in the cataphoretic mobility of the rennin from pH 6.0 to 7.5 while Richardson and Palmer (7) reported no migration at pH 6.9 to 7.0. As stated previously these workers were undoubtedly observing some less active fraction of the original rennet extract which was characterized by a coffee-brown color and an isoelectric point at pH 7.0.

DISCUSSION

The results obtained in this work show that the effect of rennin on caseinate systems is a partial destruction of both factors contributing to the stability, namely the ζ-potential and hydration. The phenomenon is not

simply a decrease in the electrokinetic charge as the results of Richardson and Palmer (7) indicate, nor is it purely a dehydration phenomenon as Söhngen, Wieringa and Pasveer (9) have postulated.

It was possible to differentiate these effects by discovering that the Krasny-Ergen (5) modification of the Smoluchowski (8) equation for electroviscous effect was applicable to experimental data on both calcium and sodium caseinate systems. Then by applying the equation to rennin treated systems, considerable information was obtained with regard to rennin action.

The data for the effect of rennin on various caseinate systems show that with low concentrations of rennin there is a large decrease in relative viscosity with very little change in the electrokinetic potential. This effect is interpreted as due chiefly to a true dehydration. As the concentration of rennin is increased the relative viscosity and ζ-potential are decreased to a critical zone of instability where several of the partially dehydrated particles clump together forming a new particle larger than the original hydrated micelle. These newly formed aggregates are still hydrated and possess sufficient charge to remain in suspension, as no visible settling out occurs.

The sensitivity of various caseinate systems toward rennin coagulation and the extent of rennin action were found to be dependent in a large measure upon the original hydration and ζ-potential. On the basis of stability the systems studied would be classified as follows: sodium caseinate > calcium caseinate > dialyzed calcium caseinate–calcium phosphate "milks" > dialyzed skim milk.

The decrease in pH at the higher rennin concentrations is due, of course, to a release of hydrogen ions. Calculation shows that this is not sufficient to account for the increased conductivity. Evidently other ions are also released but their nature is not disclosed in the experiments. The decrease in pH of rennin treated caseinate systems is only slight even when concentrated rennin is employed.

Attempts were made to study the effect of both $CaCl_2$ and rennin at low concentrations in the same calcium caseinate sol. Instability as evidenced by precipitates and clots in many cases confused the interpretation of the results. However, it appears that $CaCl_2$ destabilizes the caseinate system merely by decreasing the ζ-potential whereas the destabilizing effect of rennin is primarily one of dehydration with a secondary decrease in ζ-potential. When either destabilizing agent is present in sufficiently high concentration only a trace of the second agent is required to cause precipitation or partial clotting. This unstable zone for a 2.5 per cent calcium caseinate system at 30° C. has a ζ-potential in the neighborhood of – 10 mv., a relative viscosity of 1.20 and a pH of 6.6.

The ζ-potentials of rennin and calcium caseinate systems were found to be of the same sign and order of magnitude at the pH of milk. These results do not support the theory of Richardson and Palmer (7) that positively

charged rennin acts on negatively charged caseinate micelles at or near a pH of 6.6. There is a decrease in ζ-potential when rennin acts on the caseinate sol but the phenomenon appears to be secondary to the dehydration effect and certainly cannot be explained as an interaction of oppositely charged colloids.

This entire work has been concerned only with the primary action of rennin, and has not considered the second stage of the phenomenon, namely, the clotting. However, the application of the findings that the action of rennin is of a physical nature in destabilizing the caseinate micelles is entirely in accord with present knowledge of the second stage. This may be pictured as a coacervation or gelation of the calcium caseinate or calcium caseinate–calcium phosphate micelles, provided there are enough micelles present. In this connection it should be mentioned that during these studies firm gels were repeatedly demonstrated, even in the absence of rennin, when artificial milks were warmed to 40° C. and small increments of $CaCl_2$ added. It appears from this result that the effects of rennin are only physical in nature, since it is possible to duplicate the conditions for gel formation without rennin action. However, further experiments are necessary to determine the cause of the chemical differences between casein and paracasein described so extensively in the literature.

It is unfortunate that the electroviscosity equation does not permit an absolute evaluation of the radius and number of particles. Publication of the present work has been delayed in search of the proper equation, derived either theoretically or experimentally using model systems. The application of the correct equation, when forthcoming, to the data presented herein should further elucidate the mechanism of rennin action.

SUMMARY AND CONCLUSIONS

Data are presented for electrokinetic potential (ζ), specific conductivity (λ), relative viscosity $\left(\frac{\eta_s}{\eta_o}\right)$, and pH measurements on various caseinate systems treated with salts, rennin and pepsin. Calculations from the data are shown graphically to aid in evaluating effects on hydration and electroviscosity.

The addition of increments of rennin to calcium and sodium caseinate systems at pH 6.6 reduces the relative viscosity, ζ-potential and pH and increases the specific conductivity at varying rates and to varying degrees.

The primary effect of rennin on calcium caseinate systems appears to be a true dehydration with a secondary decrease in ζ-potential, both effects contributing to the decreased stability of the system. The effect of rennin on sodium caseinate is principally one of dehydration.

The course of action is the same for a single high concentration of rennin measured at succeeding time intervals as for a series of increasing levels of rennin measured when equilibrium is reached.

Rennin follows the same course of action on dialyzed calcium caseinate–calcium phosphate milks as it does on calcium caseinate sols even though the initial state of the former is apparently not as highly hydrated as that of the latter.

Dialyzed skim milk is an unsuitable substrate for the study of rennin action by the methods employed.

The order of destabilizing effect on a calcium caseinate sol is: Purified rennin > rennet extract > pepsin. The manner of destabilization by pepsin is somewhat different from that of rennin. The amount of pepsin in commercial rennet extract is unsufficient to influence the results obtained in a cataphoresis or viscosity study of this kind.

Rennin exhibits a progressive increase in negative ζ-potential from pH 6.0 to pH 7.5, the zone studied. Both rennin and calcium caseinate systems show the same sign and approximately the same magnitude of ζ-potential at pH 6.5.

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THE EFFECT OF DILUTION RATE ON THE LIVABILITY AND THE FERTILITY OF BULL SPERMATOZOA USED FOR ARTIFICIAL INSEMINATION

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Perhaps the most important advantage of artificial insemination over natural service in the breeding of dairy cattle lies in the increased use which may be made of superior sires by splitting of a single ejaculation of semen and the subsequent breeding of more than one cow. The number of females which may be bred by such means is dependent, among other things, on the volume of semen produced by the bull, the quality of the individual semen sample, the site of deposition of the semen in the female reproductive tract, the volume of material inseminated, whether or not the semen is diluted, and the rate of dilution employed.

Notable advances in increasing the efficiency of artificial insemination have been made during recent years through improvement in semen handling and storage techniques and through the use of improved diluents. Little attention has been paid to increasing the efficiency of the practice by determining the minimum amounts of semen required to produce pregnancies. This problem can be approached either by inseminating females with varying amounts of semen alone, by inseminating with varying amounts of semen diluted at a standard rate, or by inseminating females with a standard amount of material which may contain different amounts of semen. However, there is no certainty that deposition of a certain minimum number of spermatozoa by each of these methods would give the same results.

In routine field insemination it is essential that semen be diluted in order to increase the number of females which may be bred, and to insure maximum fertility (12). In this work it is more desirable to establish the standard dosage of diluted semen and to vary the spermatozoa dosage by means of changing the dilution rate. This is especially true where the technique of insemination used involves the fixing of the cervix per rectum and deposition of the semen directly into the uterus, as is done in the artificial breeding units of New York. Too large volumes of inseminated material may stimulate reflex contractions of the reproductive tract and lead to extrusion of the inseminated material.

Some work has been done on the problem of determining the minimum amount of semen required for successful insemination of cattle. Kozlova (5) found that 0.2 cc. of undiluted semen placed in the cervix was as effective in producing pregnancies as 4.0 cc. placed in the vagina. His data with a limited number of cows shows that when deposited in the cervix a 0.2 cc.

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dose was more effective than one of 2.0 cc. This finding supports the attitude that the volume of material inseminated should not be too large. From routine field results here it is believed that 1.0 cc. is not too large a volume for deposition directly in the uterus. However, there is some evidence at hand to show that smaller volumes may be used with success.

With respect to the influence of dilution rate on efficiency of conception, Anderson (1) in a study of two herds showed no significant difference between conception rate obtained from undiluted semen, and semen diluted up to a maximum of $\times 4$. In another herd, differences did occur, but, as he pointed out, the results for dilution rate were confounded with season, bulls, and other uncontrolled factors. With sheep both Kuznecov (6) and Anderson (2) found that fertility was markedly decreased by $\times 16$ dilution as compared to dilutions at lower rates. Panyševa (7) was able to dilute ram semen $\times 16$ with success, but increasing the dilution rate to $\times 32$ lowered the fertility.

While these results are undoubtedly of value as a guide for further work, it is questionable if they may be applied without reservation to present artificial breeding work for there has been considerable improvement in the effectiveness of diluents since the above-mentioned experiments were conducted.

It is the purpose of this paper to present the results of an extensive investigation designed to determine the effects of different rates of dilution of bull semen with yolk-citrate (9) upon its livability during storage and its fertility when used for insemination in field work after certain intervals of storage.

EXPERIMENTAL

Livability during storage. The first investigation was a laboratory study of the effects of dilution on livability. The semen was collected, handled before storage, and stored at 5° C. following the controlled-temperature techniques established in previous investigations (12). The semen was cooled to the storage temperature in steps of a 5° C. drop each 10 minutes. In the first study each ejaculate of semen was diluted with the yolk-citrate diluent at the rates of 1 part of semen to 1, 2, 4, 8, and 16 parts of the diluent.

The results of the motility observations are given in table 1. Each figure is a mean of four ejaculates. In order to prevent the varying number of spermatozoa in each microscope field from influencing the estimates of motility after storage, each sample was further diluted with a clear diluent possessing no appreciable buffer capacity (12) before motility estimates were made. By varying the amount of clear diluent added it was possible to keep the number of spermatozoa examined in each microscope field at a relatively constant level regardless of dilution rate.

Analysis of variance of the motility observations after transposition to equivalent angles by the method of Bliss (11), shows that there are significant differences ($P = < 0.01$) for the motility means between storage intervals, between semen ejaculates, and between treatments, and a significant ejaculate × treatment interaction. Apparently the livability of spermatozoa in storage decreases with each increase in dilution rate. The data for pH also are shown in table 1. They show a significant ($P = < 0.05$) change with dilution rate and for storage interval, and also show a significant interaction between ejaculates and dilution rate.

TABLE 1

Effect of dilution rate on livability of spermatozoa and on pH changes of diluted semen during storage (1st experiment)

Ratio of semen to diluter		Motility and pH means (4 ejaculates)					
		Before storage	After storage of				
			2 days	4 days	6 days	8 days	10 days
1:1	Mot. %	78	55	39	34	20	28
	pH	6.61	6.46	6.31	6.29	6.26	6.25
1:2	Mot. %	78	48	34	34	22	14
	pH	6.60	6.50	6.39	6.32	6.32	6.32
1:4	Mot. %	78	42	31	24	11	6
	pH	6.62	6.53	6.42	6.36	6.43	6.39
1:8	Mot. %	78	42	29	22	10	3
	pH	6.64	6.57	6.50	6.48	6.47	6.44
1:16	Mot. %	78	45	29	19	8	9
	pH	6.66	6.58	6.56	6.49	6.56	6.50

This study on a limited number of ejaculations of semen indicated that the ejaculates responded differently to the several dilution rates. Accordingly a more extensive factorially designed experiment involving ten separate ejaculates and two hundred and fifty separate determinations of motility and pH was undertaken. It was conducted by a different investigator than the one who made the first study. Each semen sample was diluted at the rates of 1 part of semen to 2, 4, 8, 16, and 32 parts of the yolk-citrate diluent. For motility estimates the same precautions were taken as mentioned above. The mean data for each dilution rate and each storage interval are shown in table 2. Though the semen was of somewhat better quality, and a wider dilution rate was used, the results are in complete agreement with the previous study.

Significant differences for motility means between ejaculates, between storage intervals, and between dilution rates were shown, as well as significant ejaculate × treatment and storage interval × treatment interactions. Similar significant differences and significant interactions for the pH means were shown also. The ability of spermatozoa to maintain their motility during storage is reduced with each increase in dilution rate. This fact has

been well established by this investigation, but the reason for the decrease in livability is not apparent. Whether or not it is due to a lack of substrates, a lack of accessory factors, an incompatibility between the diluent and semen, or to other factors is not known.

It is apparent, also, that the different ejaculates tend to respond differently to the several dilution rates in their livability during storage. The apparent reason for this effect lies in the difference in concentration between the several ejaculates and the correlated variations in other items of semen quality. In this investigation there was a correlation between the number of spermatozoa in the 10 ejaculates and the over-all ability of the spermatozoa to live during storage of + 0.3592. This correlation coefficient is not statistically significant. On the other hand, when the calculated number of

TABLE 2

Effect of dilution rate on livability of spermatozoa and on pH changes of diluted semen during storage (2nd experiment)

Ratio of semen to diluter		Motility and pH means (10 ejaculates)					
		Before storage	After storage of				
			2 days	4 days	6 days	8 days	10 days
1: 2	Mot. %	76	57	48	38	35	24
	pH	6.58	6.42	6.35	6.33	6.28	6.29
1: 4	Mot. %	76	54	47	37	35	29
	pH	6.63	6.50	6.44	6.41	6.35	6.34
1: 8	Mot. %	76	49	43	35	32	22
	pH	6.63	6.59	6.54	6.49	6.46	6.44
1: 16	Mot. %	76	50	41	33	26	14
	pH	6.64	6.64	6.60	6.57	6.54	6.56
1: 32	Mot. %	76	47	37	24	21	10
	pH	6.65	6.65	6.62	6.63	6.60	6.59

spermatozoa for each sample stored at each of the several dilution rates was correlated with their ability to withstand storage the correlation was + 0.4520 and highly significant.

Field studies of fertility. The storage studies indicated that for comparable results the semen samples of the highest concentration probably could be diluted to a much greater extent than the less-concentrated samples. To test this hypothesis an investigation was undertaken in cooperation with the New York Artificial Breeders' Cooperative. This organization is now (April 15, 1943) composed of 28 local artificial breeding units scattered widely over New York State. The semen used for insemination is produced by a group of about thirty bulls housed near Syracuse and shipped from that point to the member-units. The semen used in this experiment was produced by these bulls.

Following collection with the artificial vagina (10) an estimate of the concentration of spermatozoa in each ejaculate was made by standard

hemocytometer techniques, and the semen was observed for initial motility. The semen was then diluted at a predetermined rate according to the spermatozoa count and percentage motility. In general, ejaculates containing as few as 600,000 spermatozoa per mm.^{3} were diluted in the ratio of 1 part of semen to 2 parts of diluter, while semen containing 1,800,000 to 2,000,000 spermatozoa per mm.^{3} was diluted at the rate of 1 part of semen to 12 or more parts of the diluter. Semen containing less than 600,000 spermatozoa per mm.^{3} or fresh semen containing spermatozoa less than 60 per cent of which were motile was seldom used. The diluted semen was shipped to all sections of New York where it was used for breeding. One ml. of diluted semen was used for insemination. In most cases the material was placed well within the uterus at insemination.

Six and seven-tenths per cent of all inseminations were made on the day of collection, 44.6 per cent on the second day, 33.6 per cent on the third day, 10.6 per cent on the fourth day, and the remainder of the inseminations was

TABLE 3

Comparison of dilution rates when semen was diluted at rates dependent upon the concentration and the motility of the spermatozoa it contained

Ratio of semen to diluter	17 Holstein bulls			11 Guernsey bulls			All bulls		
	Services	Conceptions	% fertile	Services	Conceptions	% fertile	Services	Conceptions	% fertile
1:2	27	16	59.3	.....	.....	.....	27	16	59.3
1:3	161	81	50.3	102	32	31.4	263	113	43.0
1:4	324	161	49.7	146	84	57.5	470	245	52.1
1:5	571	282	49.4	361	224	62.0	932	506	54.3
1:6	474	232	48.9	276	154	55.8	750	386	51.5
1:7	336	180	53.6	162	100	61.7	498	280	56.2
1:8	294	151	51.4	226	139	61.5	520	290	55.8
1:9	92	56	60.9	53	30	56.6	145	86	59.3
1:10	188	92	48.9	59	37	62.7	247	129	52.2
1:12	66	40	60.6	.....	.....	.....	66	40	60.6
1:14	22	12	54.5	.....	.....	.....	22	12	54.5
Total	2555	1303	51.0	1385	800	57.8	3940	2103	53.4

made with semen stored 5 to 8 days. On the average, there were 19.3 first or second services for each semen sample used. The results are shown in table 3. The data are presented in the form of the total number of cows which were inseminated with semen diluted at each rate, and the number of cows which, during a period of at least 5 months following insemination, had not returned to service.

In the artificial breeding units cooperating in this study an agreement is made with the members that a cow which fails to conceive will be bred artificially only three times for the service fee charged. An additional charge is made for breeding one cow more than three times. Thus, cows which have been bred three times and which have failed to conceive may or may not be reported as being in calf or again coming in heat following third service.

Therefore, in this investigation services were counted only for those cows which were being bred for the first time, or for those which had failed to conceive on the first service and were being bred for the second time. Consideration was given to those cows which were bred for the third time only to determine which cows failed to conceive to the second service.

It is recognized that this method of recording fertility may overlook certain cows which actually fail to conceive on either first or second service and are either sold from the herds or otherwise disposed of before they are again bred and thus recorded as not pregnant from the previous insemination. However, though some slight error may be introduced into the records from this source there seems to be no reason to believe that such cases are not randomly distributed over the experimental treatments. In addition, the method of recording all returns to service during at least 5 months after breeding and considering such returns to be a result of the failure of the semen alone introduces some bias in the opposing direction, for some of the late occurrences of return to heat must certainly be a fault of the cow and not necessarily a fault of the semen used. The method of using all cows bred on first and second inseminations, regardless of whether or not they ever conceive, tends to reduce the conception rate figured to a somewhat lower level than where services per conception are calculated only for those cows which conceive, and where all cows failing to finally conceive are ignored.

The data presented in table 3 show that semen may be diluted at a rate based on the concentration and motility of spermatozoa with no significant trends in fertility for increasing dilution rates when used for artificial insemination. However, as should be recognized, the effects of dilution rate were sometimes confounded with bulls in this investigation for certain bulls tended to produce semen which, on the average, was different in the characteristics measured from the semen of other bulls. To obviate this disadvantage and to study further the effects of dilution rate on the fertility of semen, a more complex procedure was used in the next experiment.

The Latin square experiment. In this study it was desired to prevent all arbitrary selection of semen samples used for any particular dilution rate, and to determine the effect of the rate of dilution on the fertility of average semen samples produced by the bulls available. As trends in fertility of bulls during periods of time are sometimes observed in such work, this factor also should be eliminated as an influence in the experiment. The Latin square design (see Fisher (4)) appeared to offer the best set-up for the control of these factors. Four 6×6 Latin squares were employed. Each square was composed of 6 bulls, each with 6 dilution rates of 1 part of semen to 4, 6, 8, 10, 12, or 16 parts of the yolk-citrate diluent. Semen samples were collected from each bull approximately one week apart and for each of the six bulls one sample was taken during a particular week, this interval of

time was called the collection period. For each collection period all dilution rates were used, though each sample from a bull was diluted at only one rate. For the six collection periods the semen from each bull was diluted at each rate and for any one collection period all dilution rates were used. Thus, there were 36 ejaculates in each of the 4 Latin squares or a total of 144, with 24 used at each dilution rate. Treatments were assigned at random in each square by selection of numbered cards from a shuffled deck and the use of appropriate controls called for by the design.

The first two Latin squares made up what was called experiment 1 and were composed of two different groups of 6 bulls each, these being known as bull blocks A and B. The two groups were used in the experiment concurrently. The second experiment was a replicate of the first and the first collection of the second experiment followed within one week after the last collection of the first experiment was completed. The order of treatments was established at random in the replicate squares in order to distribute at random the possible effects of time trends in fertility of bulls.

Semen was collected and handled as previously described, save that estimates of spermatozoa count were made by means of the simple comparator recently developed here (8). In experiment 2 the methylene-blue-reduction test for quality of semen (3) also was used. All semen collected was used regardless of its apparent quality except for several first ejaculates in which the spermatozoa were completely non-motile. When this was the case, a second ejaculate was taken from that particular bull within a few minutes after the first, and this second ejaculate was used in the experiment. The lowest count observed was 566,000 spermatozoa per mm.[3] The diluted semen was shipped to the twenty or more member units of the State cooperative. Consequently, inseminations were made by twenty or more individuals. An examination of the basic data of the investigation shows that the effects of the inseminator were randomly distributed over the several bulls, treatments and collection periods. The diluted semen was generally deposited deep in the uterus, often in one or the other horn, and 1.0 ml. of diluted semen was used for insemination.

In table 4 is shown the mean data concerning the semen produced by the bulls used in this investigation. An analysis of variance of these data showed, as would be expected, significant differences between bulls in spermatozoa count, per cent of motile spermatozoa, and, in the case of experiment 2, methylene-blue-reduction time. However, no significant differences were found in these semen characteristics between means for collection periods or for dilution treatments. Thus, there were no significant differences in the semen used at the several dilution rates.

The results of the field inseminations are shown in table 5. These data were obtained in the same way as were the fertility data for the previous experiment. No trends were shown in the fertility of semen diluted at the

several rates. In fact, the highest fertility was found for the semen diluted at the rate of 1 part of semen to 16 parts of diluter. Insemination of 1 ml. of semen diluted at that rate is equivalent to 0.059 ml. of semen alone, containing on the average 74 millions of spermatozoa. The lowest conception rate was obtained from the semen diluted in the ratio of 1 part to 12 parts

TABLE 4

Mean data concerning the semen used in the Latin square experiments

Experiment	Block		Ratio of semen to diluter					
			1:4	1:6	1:8	1:10	1:12	1:16
			Actual volume semen per ml.					
			.20	.14	.11	.091	.077	.059
1	A	Count/mm.³ (thousands)	1,229	1,161	1,387	1,238	1,248	1,229
		Motility %	74	72	71	74	78	69
	B	Count/mm.³ (thousands)	1,121	1,081	1,337	1,081	1,112	1,151
		Motility %	72	78	75	75	69	72
2	A	Count/mm.³ (thousands)	1,356	1,387	1,513	1,415	1,316	1,396
		Motility %	75	82	76	80	81	73
		Methylene blue reduction test (minutes)	4.5	4.5	3.8	4.5	4.6	4.4
	B	Count/mm.³ (thousands)	1,238	1,220	1,307	1,180	1,316	1,238
		Motility %	74	74	78	79	74	77
		Methylene blue reduction test (minutes)	4.6	5.3	5.0	5.1	5.0	5.5
Mean of all		Count/mm.³ (thousands)	1,236	1,212	1,386	1,228	1,248	1,254
		Motility %	74	76	75	77	76	73

of diluter. To determine whether or not the differences observed were statistically significant an analysis of variance of the entire experiment was undertaken.

First of all, it was necessary to establish adequate methods for studying the results obtained from the single ejaculates. The lowest number of in-

TABLE 5

Fertility of the semen used in the Latin square experiments grouped by bull blocks and ratio of semen to diluter

Experiment	Block	Ratio of semen to diluter															
		1:4			1:6			1:8			1:10			1:12			1:16		
		S	C	%	S	C	%	S	C	%	S	C	%	S	C	%	S	C	%
1	A	85–36		42.4	62–28		45.2	101–41		40.6	94–50		53.2	196–86		43.9	167–79		47.3
	B	76–40		52.6	85–56		65.9	165–96		58.2	92–45		48.9	110–59		53.6	136–88		64.7
2	A	59–36		61.0	124–63		50.8	161–86		53.4	97–36		37.1	134–55		41.0	149–77		51.7
	B	69–36		52.2	60–35		58.3	117–61		52.1	122–72		59.0	125–72		57.6	137–82		59.9
Total		289–148		51.2	331–182		55.0	544–284		52.2	405–203		50.1	565–272		48.1	589–326		55.3

S = Total of 1st and 2nd services.
C = Conceptions.
% = Percentage of total 1st and 2nd services resulting in conception.

seminations per ejaculate was one, while the highest number was seventy. It is obvious that the real potential of the latter ejaculate to impregnate cows was more adequately established than was the case of the semen sample with which only one cow was bred. The most logical expression of the results would appear to be the percentage of the inseminations which resulted in pregnancies, the determination of pregnancies being made as indicated earlier. However, use of simple percentages gives equal weight to the results for each ejaculate regardless of the number of inseminations. It would appear that some weighting of the results for each semen ejaculate was necessary. On consultation with Dr. W. G. Cochran, Iowa State College, who found in another series of similar data from this laboratory that approximately 80 per cent of the within-bull variance was binomial and varied with the number of inseminations, it was decided to weight the percentage results for each ejaculate by the number of inseminations for that ejaculate.[1]

An analysis of variance of the weighted data showed no statistically significant differences between the means of dilution treatments, though significant differences were found between the means for bull blocks, and for individual bulls within the blocks. Three and nine-tenths per cent of the inseminations were made with semen used on the day of collection, 51.5 per cent on the second day, 32.7 per cent on the third day, 8.9 per cent on the fourth day, and the remainder of the inseminations was made on the fifth to eighth days following collection. Almost 100 per cent of the ejaculates at each dilution rate were used during the second and third days after collection. Following the third day the proportion of the samples which were used at each dilution rate decreased markedly, only those samples which survived storage with the greatest motility being used. No differences between dilution rates in this connection were observed. In fact, the 1:4 dilution rate was used for the shortest time following collection, 6 days. This might be considered as strange, in view of the fact that the semen diluted at the lower rates in the laboratory studies maintained greater livability on storage, if one did not recognize that less diluted semen was available for use at the lowest dilution rate and none may have been available for insemination after 6 days.

As no differences were found between dilution rates in the analysis, table 6 gives the grouped data for the entire Latin square experiment according to the day in which inseminations were made. As can be seen, there was no important deviation in fertility up to the fifth day.

DISCUSSION OF RESULTS

Because of the possibility that use of the higher dilution rates might lead to a real reduction in fertility in the Latin square experiment the most fertile bulls available were not all used in this study. The mean percentage fer-

[1] The authors are indebted to Dr. Cochran for his help with this problem.

tility for the bulls varied from 38.3 to 63.1, while the mean fertility of all semen samples used in the entire experiment was 52.0. Consequently, the mean fertility percentages for each dilution rate are primarily a reflection of the mean ability of the bulls used rather than an indication of what may be expected from the semen of other bulls used at the same dilutions.

Though there was considerable variability between dilution rates within bull blocks, there was no evidence to indicate that any trends were to be found in the results, which, because of the wide inherent differences between bulls, were impossible to demonstrate as significant. In fact, though the experimental data were obtained under conditions not entirely similar to those found in the artificial breeding units in this country, it is believed that the results are generally applicable. The conditions under which this study was carried out may be more rigorous than generally prevail, for all semen samples were shipped by parcel post before being used for insemination.

TABLE 6

Fertility of the semen used in the Latin square experiments grouped according to the interval stored before use

	Day in which insemination took place							
	1	2	3	4	5	6	7	8
Services	106	1401	890	245	54	21	4	2
Conceptions	53	751	450	124	24	10	3	0
% conceptions	50.0	53.6	50.6	50.6	44.4	47.6	75.0	0.0
% Total services each day	3.9	51.4	32.7	9.0	2.0	0.8	0.01	.....

In some cases the diluted semen was in transit as much as 24 hours before it arrived at its destination. In addition, practically all ejaculates were used for three days after collection, and some were used for a considerably longer period.

On the average 18.9 cows were bred with each ejaculate used in the Latin square experiment. As can be seen in table 5 the number of cows inseminated with semen diluted at the ratio of 1:16 was slightly more than double the number inseminated with semen diluted at the ratio of 1:4. Theoretically semen would be available to breed over three times as many cows. The actual volume of semen used varied from 0.2 ml. at the lowest rate of dilution down to 0.059 ml. for the highest rate. The average number of spermatozoa per insemination varied from about 247 millions down to about 74 millions.

This investigation was carried out during March, April, and May, when the number of cows available for service was limited. During periods of the peak in breeding, the late fall and winter months, many more cows could have been bred, especially with semen diluted at the higher rates. These facts indicate that bull semen of good quality may be diluted at higher rates

than generally used without decreasing its effectiveness, providing insemination is properly carried out, and that many more cows may be bred with each satisfactory ejaculate than is now being done. During peak seasons of breeding the use of increased rates of dilution should enable artificial breeding units not only to breed more cows with the semen of each bull, but, also, to collect semen from each bull less frequently than would otherwise be necessary, and, thus, keep outstanding proved sires in service for a maximum period of time.

SUMMARY

Bull spermatozoa diluted with yolk-citrate diluent showed a decreased ability to maintain their motility during storage at 5° C. with each increase in rate of dilution. Dilution rates of from 1 part of semen to 1 part of diluter, up to 1 part of semen to 32 parts of diluter were used. Semen possessing higher numbers of spermatozoa, a large percentage of which were actively motile on initial examination, survived storage at the higher dilutions better than did semen of lower spermatozoa count and lower initial motility.

In field studies, when the semen was diluted at a predetermined rate based on the spermatozoa count and the percentage of motile spermatozoa in each semen sample, no differences were found in fertility between semen diluted at the rates of 1 part of semen to 2 parts of diluter up to 1 part of semen to 14 parts of diluter. Over 3900 inseminations were involved. In an investigation designed to eliminate arbitrary selection of the rate of dilution to be used for any satisfactory semen sample, no difference was found in fertility for semen diluted at the rates of 1 part of semen to 4, 6, 8, 10, 12, or 16 parts of diluter. Semen at all dilution rates was used for as long as 6 to 8 days after collection. No observable differences were noted in the maintenance of fertility for the several dilution rates during the first 4 days of storage. For diluted semen used longer than this period, the number of inseminations was too few on which to base conclusions.

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ANNOUNCEMENT

August 26, 1943

OFFICERS AND MEMBERS OF THE AMERICAN DAIRY SCIENCE ASSOCIATION:

Members of the Ohio State University staff are delighted that the American Dairy Science Association has accepted an invitation to hold its annual meeting on our campus next June 20, 21, and 22.

We recall with pleasure your previous meeting at Ohio State in 1938. Your presence at that time was stimulating to our faculty and it is our hope that your visit was equally helpful to the association.

Ohio State University and all of its personnel pledge their very best efforts to make your 1944 meeting all that you want it to be, and more. We recognize the vital role the dairy scientist plays in these times of war, and also the great responsibility he faces in the years of reconstruction.

It is our hope that the June meetings at Ohio State will make an important contribution in helping to solve your war and post-war problems.

Sincerely yours,

HOWARD L. BEVIS, *President*

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ABSTRACTS OF LITERATURE

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MILK AND MILK PRODUCTS

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CONTENTS

Bacteriology
Breeding
Butter
Cheese
Chemistry
Concentrated and dry milk; by-products
Diseases
Feeds and feeding
Food value of dairy products
Herd management
Ice cream
Milk
Miscellaneous
Physiology

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Lancet
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Milk Industry
Milk Plant Monthly

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ABSTRACTS OF LITERATURE

BACTERIOLOGY

493. The Streptococci Present in the Feces of Patients with Non-Specific Ulcerative Colitis, and the Effect of the Oral Administration of Sulfonamide Compounds upon Them. ENID C. RODANICHE, WALTER LINCOLN PALMER, AND JOSEPH B. KIRSNER, Univ. of Chicago. Jour. Infect. Dis., *72*, No. 3: 222–227. May–June, 1943.

Of 216 strains of nonhemolytic streptococci isolated from feces, 29 strains from normal persons and 187 from persons with chronic ulcerative colitis or other related conditions, 151 were classified as *Streptococcus fecalis*, 9 as *Str. liquefaciens*, 2 as *Str. equinus*, 11 as *Str. bovis* (variant), 20 as *Str. salivarius*, and 23 were unclassified. Oral administration of sulfasuxidine, sulfaguanidine, sulfadiazine, or sulfathiazole to the patients resulted in changing the flora in the intestines from one of predominantly Gram-negative coliform bacilli to one of predominantly Gram-positive cocci, usually streptococci. The administration of the sulfonamides did not cause any alteration of the types of streptococci present in the feces. J.F.C.

494. Notes on the Use of Sodium-Lauryl-Sulfate Tryptose Broth for the Detection of *E. Coli* in Milk. MARY A. GARDNER, ORVILLE W. GARRETT, AND PAUL S. PRICKETT, Mead Johnson and Co., Evansville, Ind. Jour. Bact., *45*, No. 4: 413. Apr., 1943. Abs. Proc. Local Branches.

Sodium-lauryl-sulfate tryptose broth exhibited undesirable physical characteristics; while it appeared to be as sensitive for the detection of *E. coli* in fluid and dried milk as brilliant-green bile broth the latter produced significantly less false presumptives. D.P.G.

495. Factors Which Influence the Growth of Heat-Treated Bacteria. I. A Comparison of Four Agar Media. F. E. NELSON, Kans. Agr. Expt. Sta. Jour. Bact., *45*, No. 4: 395–403.

The apparent survival of heat-treated bacteria can be varied by the use of different media; relative suitability of the different media varies with the test organisms. The use of beef-infusion agar resulted in the enumeration of viable organisms of most of the heat-treated thermoduric lactic streptococci. Bacteria which have been subjected to heat at partially lethal levels are more demanding in the requirements of media for growth than are unheated organisms. D.P.G.

496. Application of the Decimal Reduction Time Principle to a Study of the Resistance of Coliform Bacteria to Pasteurization. LEONARD

I. KATZIN, Univ. Rochester, School of Med. and Dentistry; LESLIE A. SANDHOLZER AND MARY E. STRONG, Natl. Inst. of Health. Jour. Bact., *45*, No. 3: 265–272. March, 1943.

As an aid in quantitative determinations of bacterial death by heat, the authors have proposed the concept of "decimal reduction time" which is based upon the application of the monomolecular reaction rate constant to bacterial death under uniform conditions. DRT was obtained for 66 strains of *Escherichia* from various sources. The data indicate that very few coliform bacteria ever survive pasteurization; pasteurized milk showing numbers of coliforms has been subjected to post-pasteurization contamination. A table presents DRT for a variety of other bacteria in milk calculated from data in the literature. The methods of determining the margins of safety of a pasteurization process are noted. It is suggested that DRT be included as one of the standard criteria for determining the efficacy of pasteurization and similar processes. D.P.G.

CHEESE

497. Quality Specifications for Lend-Lease Cheese. H. L. WILSON, U. S. Dept. Agr., Washington, D. C. Natl. Butter and Cheese Jour., *34*, No. 8: 38. Aug., 1943.

Cheese for lend-lease should be of U. S. No. 1 quality. Defects can be avoided by milk grading, keeping sanitary factory, and by carefully making, paraffining, boxing and storage. W.V.P.

498. Synthesis of Vitamins by Microorganisms in Relation to Vitamin Content of Fancy Cheeses. P. R. BURKHOLDER, JANE COLLIER, AND DOROTHY MOYER, Osborn Botanical Lab., Yale Univ., New Haven, Conn. Food Res., *8*, No. 4: 3. July–Aug., 1943.

Studies indicated that common microorganisms comprising the surface flora of four kinds of fancy cheeses were capable of synthesizing important quantities of B vitamins in synthetic media. Microbiological assays seemed to indicate that thiamin, riboflavin, biotin and niacin increased in the surface layers of Brie, Cammembert, Liederkranz and Limburger cheeses during ripening as a result of synthesis by microorganisms. On the other hand, the subsurface layers and the cores of these cheeses, in general became depleted in these vitamins as curing progressed up to 39 to 44 days.

F.J.D.

CONCENTRATED AND DRY MILK; BY-PRODUCTS

499. A Colorimetric Method for the Determination of Fat-Peroxides and Its Application in the Study of the Keeping Quality of Milk

Powders. R. A. CHAPMAN AND W. D. MCFARLANE, Macdonald College, Quebec. Canad. Jour. Res., *21*, B,7: 133. 1943.

A colorimetric method based on the oxidation of ferrous to ferric iron and the determination of the latter as ferric thiocyanate has been found suitable for the estimation of fat-peroxides in milk powder. To an acetone extract of the milk powder is added a solution consisting of 0.1% of ferrous ammonium sulphate and 0.4% of ammonium thiocyanate in 96% acetone, and the color developed by heating. The intensity of the red color is measured with a Coleman spectrophotometer and is found to bear a close relation to the keeping quality of the milk powder. Peroxide values determined by this method are considerably higher than those obtained by an iodimetric procedure. O.R.I.

DISEASE

500. A Milk-Borne Epidemic of Brucellosis Caused by the Porcine Type of Brucella (*Brucella suis*) in a Raw Milk Supply. I. H. BORTS, State Hygienic Lab., Iowa City, Ia.; D. M. HARRIS, M. F. JOYNT, J. R. JENNINGS, AND C. F. JORDAN. Jour. Amer. Med. Assoc., *121*: 319. Jan., 1943.

In an epidemic of brucellosis 77 persons were found to show positive clinical or agglutination findings caused by *Brucella suis*. All received raw milk from a common dairy. Blood cultures were made from 29 patients and *Br. suis* was isolated from 13, or 45%. Of 43 dairy cows on the farm 3 were definitely positive reactors, 1 was suspicious. *Br. suis* was isolated from the milk of 2 reactors on direct culture of gravity cream. Guinea pig inoculation resulted in the isolation of *Br. suis* from 3 reactors. Hogs and dairy cows mingled freely on the farm. Of 24 sows tested there were 11 positive and 3 suspicious reactors. There was no evidence of frank swine abortion.

One human patient, an expectant mother with one healthy child, acquired brucellosis in moderately severe form, being confirmed by a positive agglutination test. This patient aborted twin embryos during the fourth or fifth month of gestation; there was no other discernible cause for the abortion.

All 6 members of one family and, in another home, 6 of a family of 12, suffered attacks of brucellosis. D.P.G.

501. The Occurrence of Food-Poisoning Staphylococci in Pasteurized Milk. KATHRYN CHALLINOR, M. SCHERAGO, AND R. H. WEAVER, Univ. of Kentucky. Jour. Bact., *45*, No. 6: 577. June, 1943. Abs. Proc. Local Branches.

Twenty samples of pasteurized milk were examined for presence of food-poisoning staphylococci. Of 18 isolates tested three were found to be

enterotoxin producers. All three strains came from milk from one plant. Thermoduric studies revealed that two strains resisted 65° C. for 20 minutes and one strain resisted 85° C. for 20 minutes. D.P.G.

502. Staphylococcus Intoxication with Two Fatalities Due to Milk from a Goat Suffering from Staphylococcus Mastitis. L. A. WEED, A. C. MICHAEL, AND R. N. HARGER, Ind. Univ. School of Med., Indianapolis. Jour. Bact., *45*, No. 4: 414. April, 1943. Abs. Proc. Local Branches.

Two children and one adult developed acute gastro-intestinal symptoms with marked vomiting and diarrhea 1.5 hours after drinking raw milk taken from a goat which died within 24 hours after the last milking. The children died about 18 and 24 hours after drinking the milk. One half of the udder of the goat was the site of an acute suppurative mastitis with numerous abscesses opening into the lactiferous ductules. Microscopic examination and cultures of material taken from the udder revealed enormous numbers of *Staphylococcus aureus*, a culture filtrate of which produced typical enterotoxic symptoms and death in experimental animals. D.P.G.

503. Staphylococci in the Bovine Udder. WILLIAM T. MILLER, Anim. Dis. Sta., Beltsville Res. Center, U. S. D. A. Jour. Bact., *45*, No. 3: 307. March, 1943. Abs. Proc. Local Branches.

Staphylococcus aureus was found in 33.3% of milk samples from 445 cows in 6 dairy herds. In 2 herds the number of infected cows exceeded 60%. Persistent infection occurred in certain cows in 3 herds for at least 12 months. Seven strains of *Staph. aureus*, 6 of bovine and one of human origin, were used to expose 26 quarters of 8 cows by way of the teat canal on 55 occasions. Infection occurred in 22 quarters and in one cow all 4 quarters were infected with the human strain. Staphylococcal antitoxin appeared in the blood following udder infection but none was found in the milk from undamaged quarters. Antitoxin was present in the colostrum but disappeared as soon as the secretion assumed the appearance of normal milk. D.P.G.

504. Brucellosis: Consideration of Its Epidemiology, Diagnosis and Control. CARL F. JORDAN, Epidemiologist, Iowa State Dept. Health; IRVING H. BORTS, Assoc. Dir., State Hygienic Lab., Iowa City; DONALD M. HARRIS, Med. Dir., Dist. Health Serv. No. 3, Spencer; AND JAMES R. JENNINGS, Milk Sanit., Iowa State Dept. Health, Des Moines, Ia. Amer. Jour. Pub. Health, *33*, No. 7: 773–779. 1943.

Information on milk-borne brucellosis which is presented is based on an investigation of two cases caused by *Brucella abortus* in Madrid, Iowa, and

on an epidemic in Marues, Iowa, caused by *Brucella suis*. Results of agglutination and skin tests of users of the contaminated supplies are compared with tests of users of "other milk supplies." In the case of the Marcus outbreak, among 57 persons of all ages who used the contaminated milk supply, 73.7% showed positive skin reactions, whereas only 10.8% of 158 consumers of other milk showed a positive or an allergic response. Diagnosis, control and prevention are discussed briefly. M.W.Y.

505. A Combined Outbreak of Septic Sore Throat and Diphtheria. G. GRAHAM SIMMS, Dept. of Pub. Health, Nova Scotia. Canad. Pub. Health Jour., *34*, No. 4: 152–157. 1943.

An investigation is reported of this combined epidemic in a university residence in which four cases of diphtheria and more than one hundred cases of septic sore throat occurred. Raw milk was consumed at the institution and the evidence suggests that one cow suffering from mastitis and shedding hemolytic streptococci in her milk may have been the source of the infection. O.R.I.

506. Bacterial Food Poisoning. C. E. DOLMAN, Univ. of Brit. Columbia, Vancouver, B. C. Canad. Pub. Health Jour., *34*, No. 3: 97. 1943, and *34*, No. 5: 205. 1943.

This article reviews much of the recent literature on this subject and in addition presents many of the author's findings on staphylococcus food poisoning and the enterotoxins produced by these organisms. An increased incidence of food poisoning is to be anticipated as a result of wartime conditions. Human and animal Salmonella carriers are of higher incidence than is often supposed and it is now known that some Salmonella organisms formerly thought to be animal pathogens only are potentially pathogenic for man. It is doubtful if food poisoning can be produced by the ingestion of the toxins of the Salmonella group, however.

Enterotoxigenic staphylococci account for the most prevalent type of food poisoning. The reactions of human volunteers who took filtrates from staphylococcus cultures are described. None of these developed any apparent immunity by repeating the experience four times. Intravenous administration of filtrates to kittens is also used as a diagnostic test. Feline vomiting reactions were also provoked by intraperitoneal and intravenous injections of filtrates from single strains of *B. coli* and *Proteus vulgaris*, although human volunteers took up to 50 ml. of these and other filtrates without harmful effects.

Some types of bacterial food poisoning due to enterogenic staphylococci may appear to be due to other types of organisms largely because the staphy-

lococcus infection has been masked or overgrown by these other types by the time incriminated food reaches the laboratory for examination.

O.R.I.

ICE CREAM

507. Ice Cream Consumption and Income. ANONYMOUS. Ice Cream Field, *41*, No. 5: 24. 1943.

Mention is made of the report of the Bureau of Agricultural Economics in which it is stated that the amount of ice cream consumed varies in proportion to the consumers' income. This is followed by a report of a study of consumer habits for ice cream made by the Milwaukee Journal.

It is stated that in Greater Milwaukee, of the total families paying $30.00 or less a month rent, 52.1% regularly ate bulk ice cream; of the families paying $30.00 to $40.00 a month rent, 55.7% regularly consumed bulk ice cream; of those paying $40.00 to $50.00 a month rent, 58.3% regularly had ice cream in this form, while of those paying $50.00 and over in rent, 62.2% regularly enjoyed bulk ice cream. This shows that people in the low income groups are large and regular consumers of ice cream, it is stated.

For these same income groups, the percentages of the families which regularly consumed brick ice cream was 82.3%, 82.2%, 82.3% and 84.3%, respectively. "In the final analysis," it is concluded, "everybody likes ice cream, and there is hardly a person or family that cannot afford to eat it regularly." W.C.C.

508. Feasibility of Processing Vegetables, Fruits in Ice Cream Plants Previous to Freezing. J. M. LAMBERT, Temporary Staff Res. Engin., Dairy Indus. Supply Assoc. Ice Cream Field, *41*, No. 6: 16. June, 1943.

The consensus of opinion was that fruits and vegetables could best be handled near points of harvest by existing equipment, principally by the canning industry. The following reasons for this point of view are listed: (1) Ice cream plants large enough to quick freeze significant quantities of material are generally located in large cities. In most such cases space is inadequate for the handling of raw stock and trucks required for transportation. (2) Ice cream manufacture is basically an intensive manufacturing process carried out in limited space; hence, processing previous to quick freezing is incompatible with ice cream plant design. (3) Ice cream plants are primarily designed to handle liquids in pipelines under gravity or pump pressure. The processing of fruits and vegetables necessitates the use of conveyors, belts, worm gears and chain bucket drags. Ice cream equipment is therefore not suitable for this purpose. (4) Water supply would be

inadequate for washing of fruits and vegetables in most ice cream plants. (5) Officials of existing legislation governing the operation of ice cream and dairy plants would oppose the use of dairy equipment for the processing of fruits and vegetables. (6) The blanching of raw fruits and vegetables would require only a part of the boiler capacity in the average ice cream plant, hence, would result in uneconomical use of fuel. (7) Wartime restrictions on replacement of ice cream equipment necessitate its conservation and limited use. (8) Transportation of inedible portions of fruits and vegetables from point of harvest to city plants and subsequent disposal of wastes would be expensive. (9) It would be difficult for workers in the ice cream industry to acquire the necessary skill to process fruits and vegetables when they are already working to full capacity. (10) Excess refrigeration in sufficient amount will not be available unless ice cream manufacture is further restricted. W.C.C.

509. Suggestions on the Use of Casein in Ice Cream. THEO. R. FREEMAN, Fla. Agr. Expt. Sta., Gainesville. Ice Cream Field, *41*, No. 5: 14. May, 1943.

Preparing the casein by precipitation with dilute hydrochloric acid is described. It consists essentially of adjusting the acidity of skim milk to 0.47% to 0.50% and, after draining, washing the curd with acidified water.

The casein can be held at 45° F. 3 to 5 days, at 35° F. 10 days, and at temperatures "well below freezing" it may be kept for a year. Trials in which sodium propionate (Mycoban) was used as a preservative showed that the addition of 0.2% made possible storage for 3 to 6 weeks at 35° F. and 3 to 4 weeks at 45° F. The use of 0.4% Mycoban appeared to be no more effective than 0.2% in preventing mold growth, but it did delay the development of putrid odors.

Not more than 35% to 40% of the serum solids in normal (10% to 11% serum solids) ice cream should be derived from casein. The following objections are given to using casein to excess: (1) The mix whips so rapidly that overrun control is difficult, (2) The flavor of the ice cream is impaired, and (3) There is a tendency towards a chewy or pasty body. The necessity of knowing the composition of the casein preparation is emphasized.

The recommended method of incorporating the casein into an ice cream mix is first to prepare a 20% "casein gel" by mixing the casein in sufficient water to which has been added about 0.25 pound of baking soda for each 10 pounds of curd used. Several hours are required to form the gel after which it can be added to the balance of the mix at 120° F. as it is being heated for pasteurization, otherwise the mix is processed in the usual manner.

Teichert *et al.* are quoted as saying that excessive overrun can be prevented by reducing to one-half the amount of stabilizer used and by the addition just before pasteurization of 30 grams of anhydrous calcium chloride per 100 pounds of mix. W.C.C.

510. Dried Whole Egg Powder. II. Effect of Heat Treatment on Quality. W. HAROLD WHITE AND M. W. THISTLE, Natl. Res. Council, Ottawa, Canada. Canad. Jour. Res., *21*, D,7: 194. 1943.

The effect on quality of heating dried egg powder at temperatures ranging from 26.7° to 60° C. for from 3 hours to 7 days was investigated.

Quality deterioration was assessed by determining the fluorescence, potassium chloride and water value and the pH and refractive indices of the potassium chloride and aqueous extracts.

The rate of quality deterioration was on the average usually greatest on heating for one day at temperatures of 43.3° C. or higher. The results indicate that egg powder should be cooled after drying so that the temperature reaches 26.7° C. or less within 3 hours. O.R.I.

511. Dried Whole Egg Powder. III. A Refractometric Method for the Determination of Solubility. W. HAROLD WHITE AND G. A. GRANT, Natl. Res. Council, Ottawa, Canada. Canad. Jour. Res., *21*, D,7: 203. 1943.

Egg powder of edible quality may be divided into those suitable for consumption in egg dishes and those satisfactory only for use by bakers and other food manufacturers requiring a source of edible, soluble protein for their products. A method is described for determining the "refractometric value," which is indicative of the soluble protein content of the powder. The powder is defatted with petroleum ether and then extracted with a 5% solution of sodium chloride, after which the refractometric value of the extract is determined using an Abbé refractometer. O.R.I.

512. Dried Whole Egg Powder. IV. Effect of Moisture Content on Keeping Quality. W. HAROLD WHITE AND M. W. THISTLE, Natl. Res. Council, Ottawa, Canada. Canad. Jour. Res., *21*, D,7: 211. 1943.

Dried whole egg powders obtained from three different manufacturers were adjusted to contain from 2 to 8.5% moisture, and held at temperatures ranging from 7.1° to 43.3° C. Quality was assessed by determination of the fluorescence, potassium chloride and refractometric values.

Temperature was the most important single factor studied affecting the keeping quality of dried egg. However, at all temperatures the rate of

deterioration increased with increases in moisture content. To maintain quality during storage, dried egg should not contain more than 5% moisture and preferably 2% or less. O.R.I.

MILK

513. Laboratory Control of Milk Under War Conditions. The Rapid Phosphatase Test. H. SCHARER, Chem. Lab., Dept. of Health, New York, N. Y. Amer. Jour. Pub. Health, *33*, No. 4: 396–398. 1943.

The importance of the careful preparation of reagents and use of adequate controls are stressed. The unavailability of normal butyl alcohol prompted a further modification of the rapid phosphatase test which is midway between the earlier "field test" and the laboratory procedures, and which can be utilized equally well by the laboratory or the inspector. The name "control test" is suggested. When time is no great factor it is suggested that the incubation period be extended to one hour, the precipitation technique dispensed with and the dibromoquinonechloromide added directly when the test becomes sufficiently sensitive for all practical purposes.

In the application of any of the techniques to high-temperature short-time pasteurization, it is necessary to have some information regarding the preheating time and temperature as well as the rated holding time and temperature. In the case of cream, it is frequently necessary to have some knowledge of the history, particularly the age of the sample after pasteurization, before a sample may definitely be classed as improperly pasteurized. M.W.Y.

514. Laboratory Control of Milk Under War Conditions. WALTER D. TIEDEMAN, State Dept. of Health, Albany, N. Y. Amer. Jour. Pub. Health, *33*, No. 4: 401–403. 1943.

The use of the phosphatase test to determine adequate heat treatment or pasteurization of the milk, the coliform test to show possible contamination after pasteurization, and the direct microscopic count to indicate the bacterial content, in a general way should give the health officer the information he needs to know about a milk supply. The substitution of the direct microscopic count for the standard plate count in the examination of pasteurized milk and cream offers possibilities for wartime conservation of time and materials. Adoption of a 200,000 direct microscopic clump count limit on pasteurized milk used in conjunction with the phosphatase test and the coliform test should enable health officials to maintain adequate public health control of such milk supplies without the use of the standard plate count. M.W.Y.

515. Laboratory Control of Milk Under War Conditions. Milk Testing Under Emergency Conditions. R. V. STONE, Los Angeles, Calif. Amer. Jour. Pub. Health, *33*, No. 4: 399–400. 1943.

The Pearl Harbor treachery stimulated early action in Los Angeles County to formulate a Dairy Industry Emergency Civilian Defense General Committee. Twelve committees were set up. The Technical Committee was assigned, primarily, to laboratory and associated problems. Plans for laboratory work in case of an emergency are discussed. M.W.Y.

516. Laboratory Control of Milk Under War Conditions. C. K. JOHNS, Dept. Agr., Ottawa, Canada. Amer. Jour. Pub. Health, *33*, No. 4: 393–395. 1943.

A quality control program may be carried out with simplified laboratory procedures which make less demand upon skilled help, apparatus, and supplies. The "triple reading" resazurin test has definite advantages for the grading of milks on the basis of both bacterial and leucocyte contents, while the oval tube method is preferred following laboratory pasteurization for the detection of milks containing thermoduric organisms in significant numbers. M.W.Y.

517. Laboratory Control of Milk Under War Conditions. A. W. FUCHS, U. S. Pub. Health Serv., Washington, D. C. Amer. Jour. Pub. Health, *33*, No. 4: 390–392. 1943.

Milk laboratory surveys in defense areas have been made throughout the country to determine whether equipment and procedures employed in bacteriological examinations are in conformity with the Standard Methods for the Examination of Dairy Products, and to suggest improvements toward that goal. The survey indicates the need for an adequate system of state approval or licensing of bacteriological milk laboratories. The need for speedy tests, the results of which are available in time to keep unsafe milk off the market, is stressed. M.W.Y.

518. Simplification of Laboratory Control Procedures for Market Milk. ROBERT S. BREED, N. Y. Agr. Expt. Sta., Geneva, N. Y. Amer. Jour. Pub. Health, *33*, No. 4: 386–389. 1943.

The desirability of basing the entire milk control program on tests of a type that can be applied by the inspector on the spot with subsequent rejection or embargo placed on the milk before use, is emphasized. The odor test with rejections checked by microscopic examination is recommended as used by the New York City Inspection group. Procedures useful in judging the quality of bottled pasteurized milk are discussed. M.W.Y.

519. Trends in Laboratory Procedures in the Milk Control Program of St. Louis. JANE HERSHEY, HARRY WALDRIP, AND JOSEPH C. WILLETT, St. Louis Health Div. Labs, St. Louis, Mo. Amer. Jour. Pub. Health, *33*, No. 4: 339–342. 1943.

The methylene blue test for raw milk and the phosphatase test for pasteurized milk are discussed. A simplified procedure is used for the methylene blue test. A pipetting machine is used to add the methylene blue. The tubes, which are in racks holding 10, are stoppered with a strip of smooth-surfaced sponge rubber, long enough to cover the rack, held in a metal gripper. This is held so tightly against the mouths of the tubes that they may be inverted and shaken vigorously without leakage. As one rubber grid is used for shaking, an assistant rinses a second in running tap water and blots it with a fresh paper towel. Two people can set up 1,500 or more tests in about an hour. Only half as many samples are now reduced in less than 6 hours as in 1938. M.W.Y.

520. Milk Laboratories in War Areas. LUTHER A. BLACK, Sanitation Sect., States Relations Div., U. S. Pub. Health Serv., Washington, D. C. Amer. Jour. Pub. Health, *33*, No. 7: 824–832. 1943.

In the interest of increasing the accuracy of bacteriological milk analysis, 281 laboratories in 40 states were surveyed. Of these, 276 used the agar plate method. Compilations were made of the number of laboratories conforming to, and deviating from, each item of equipment, preparation, technique, and reporting required by Standard Methods. No laboratory conformed in all items of even the general group on apparatus, or technique. One laboratory conformed in preparation, one in incubation, 11 in counting and 25 in reporting results. The U. S. Public Health Service survey forms should be of value to administrators in improving the work of laboratories in their jurisdiction. M.W.Y.

521. Producing Quality Milk. EARL WEAVER AND J. M. JENSEN, Mich. State Col., East Lansing. Mich. Agr. Ext. Bul. 245. 1943.

This publication outlines the essentials for high quality milk production and describes common methods used for testing milk quality. P.H.T.

522. Credit Problems in Wholesale Milk Areas of New Hampshire. BYRON PETERSON, N. H. Agr. Expt. Sta., Durham. N. H. Agr. Expt. Sta. Cir. 64. 1943.

One hundred dairymen were interviewed concerning the amount, cost, and type of their indebtedness. Of these, 79% used credit, their liabilities equalling 21% of their assets. Short term loans were secured principally

from local banks. Loans for intermediate periods of time were secured principally from the Farm Security Administration and the Production Credit Association. Of the 60 long term loans, 26 were made by local banks, 23 by the Federal Land Bank, and 11 by individuals. Interest rates were not excessive. Through the wise use of credit, many dairy farms could be developed to fuller capacity. Lending agencies need to become better acquainted with farmers and better informed as to the individual and collective problems of farm borrowers. P.H.T.

523. An Easy Method for Determining Total Solids in Milk. LEOPOLDO L. RUEDA, Havana, Cuba. Natl. Butter and Cheese Jour., *34*, No. 8: 12. Aug., 1943.

A nomograph is described for determining quickly the total solids in milk by the Sharp and Hart formula when the lactometer reading at 30° C. (86° F.) and the fat content of the milk are known. For calibrating the lactometer to be read at 30° C., six and ten per cent solutions of cane sugar are used which have specific gravities at 30° C. with reference to water at 30° C. of 1.02351 and 1.03982, respectively. Differences from these specific gravities indicated by the lactometer readings of these two solutions at 30° C. furnish the desired adjustment factor for using the lactometer at this temperature. Instructions are given for determining the correction in lactometer reading for slight deviations of temperature from 30° C. The nomograph is printed large enough so that it can be mounted and used in plant or laboratory practice. W.V.P.

MISCELLANEOUS

524. Some Characteristics of Refrigerants. BENJAMIN H. SPURLOCK, JR., Univ. of Colo., Boulder, Colo. Refrig. Engin., *46*, No. 2: 107. 1943.

The author lists the properties of the ideal refrigerant as being: 1. High latent and high ratio of specific heat of vapor to specific heat of liquid. 2. A boiling point at atmospheric pressure low enough to obtain the desired temperature. 3. A relative low pressure at the cooling medium temperature. 4. A low ratio of compression. 5. A low specific volume of the vapor leaving the evaporator. 6. A high critical temperature; low freezing temperature. 7. Non-corrosive action on metals, rubber and bearing materials. 8. A chemical composition which is stable under working conditions and inert on lubricants. 9. A non-inflammable and non-explosive nature, even when mixed with air. 10. An inoffensive odor. 11. A non-toxic nature even when mixed with air. 12. Low cost of production. 13. A behavior whereby its presence in small quantities may be visibly detected by a simple

test. 14. Low viscosity, gas and liquid. 15. High conductivity (heat) and film coefficient.

A table accompanies the article which has been compiled from many sources listing the characteristics of 24 refrigerants under 42 headings, making the most comprehensive refrigerant comparison chart ever prepared and published. L.M.D.

525. Some Factors Influencing the Toxicity of Ozone to Fungi in Cold Storage. R. D. WATSON, Dept. of Plant Pathology, Cornell Univ., Ithaca, N. Y. Refrig. Engin., *46*, No. 2: 103. 1943.

The experimental work described was applied primarily to apple storage. Advantages of ozone as a fungicide agent in cold storage are: 1. Ozone leaves no residue except oxygen. It may be safely used as a fungicide without fear of a harmful residue. 2. Since ozone is a gas it gives fungicidal action wherever air is able to diffuse readily. Ozone, therefore, reaches the apples and inhibits mold growth within and throughout the package in places that would be difficult or impossible to reach without the use of a gaseous fungicide. 3. Since ozone is used as a fungicide at relatively low concentrations, of the order of 0.4 to 2 parts per million, and is constantly being replaced, it is unnecessary to have an airtight or sealed storage. The room may be entered freely without appreciably affecting the ozone concentration in the room. 4. Many of the advantages of the use of ozone are associated with its oxidizing power. The oxidation and destruction of any odor-producing substances leave the treated room free of odors such as those from fruit, boxes, and fungi. 5. At the concentrations used in apple storage rooms, up to one or two parts per million, no ill effects were observed on persons entering the rooms.

The limitations are: 1. Ozone is unstable and must be used continuously or intermittently at regular intervals. 2. Ozone oxidizes some substances more rapidly than does oxygen. It is inactivated by rubber and likewise causes damage to the rubber. Fatty substances become rancid in ozone storage more rapidly than in storage without ozone. (*Abstractor's observation: Butter, Cheddar cheese, cream and Neufchatel cheese should not be exposed to ozonization, because surface oxidation will occur.*) 3. Organic material like the exposed flesh of fruit destroys ozone, thus limiting its effectiveness in protecting cut or punctured apples from rotting. 4. The low concentrations of ozone used limits its rate of diffusion so that it becomes ineffective in controlling mold or bacterial growth throughout liquids which have considerable depth. 5. Ozone in relatively high concentrations causes minor injury to soft types of fruit. L.M.D.

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The Indians of the 16th Century observed that certain changes in the ripe beans on the ground produced the fascinating aromatics. With typical Aztec ingenuity, they decided that a better result could be obtained by spreading the beans out in some *natural* sunny area. From this elemental beginning, great strides have been made in the essential curing which must take place to produce any semblance of vanilla flavoring.

About a century ago, someone introduced the idea of *artificial* heat. In Madagascar, the French developed the method of dipping the beans in hot water to help the enzymes start their chemical work within the bean. In Dominica and Guadeloupe, the curers scratch the beans with a needle, then string them on a line to be developed by the sun. Today, experiments are being carried on by the Agricultural Experiment Station in Puerto Rico with ethylene and other gases; with electric ovens as a source of artificial heat; with man-made refrigeration as a means of breaking down the cell walls to help the enzymes go to work.

Every curing method results in some advantages and some disadvantages. For instance, the Mexican method secures benefits from the extreme amount of sunning which is required. However, this very sunning drives off some of the invaluable volatile vanillin. Other methods achieve some advantages but fail to develop as much fragrance.

It was natural that the men who have devoted a lifetime of interest and study to all phases of the production of this wonderful flavoring, learned some of the chemical secrets of the vanilla bean. They found that from the start of the curing to the finished package of vanilla flavoring, a series of related chemical actions must take place. By artificial means, Man helps the enzymes, within the bean, to do their best work in synthesizing the flavoring compounds. When the enzymes have gone as far as they can, the laboratory takes over and completes the job of bringing the ultimate flavoring to the peak of balance. There is no place where it can be said Nature stops and Man begins. There must be teamwork from the very beginning.

Just what constitutes the finest vanilla flavoring offers a subject for interesting discussion. In this land where we like to reduce everything to a formula or a brief definition, the problem of vanilla flavoring stumps the experts. It is unrealistic to classify the flavoring compounds of the properly cured vanilla bean as "natural" because so many artifices are required in their present-day production. It is unrealistic to classify vanillin as "artificial" because the compound occurs so frequently in Nature. And it is unrealistic to classify the combination of the two as "artificial" or "natural." And so, in the present high development of food products, it seems as if the words "natural" and "artificial" have outworn their usefulness.

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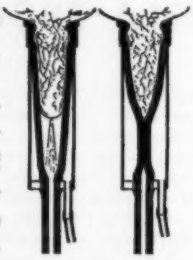

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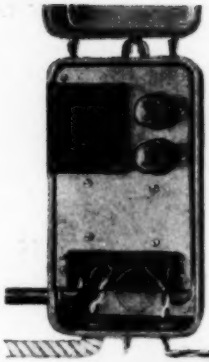

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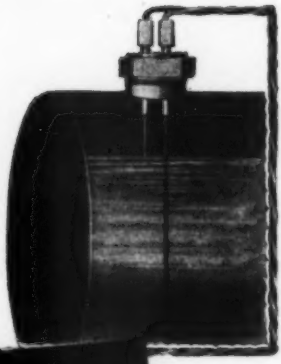

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